Utility of loop-mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis: A pilot study

Dissertation submitted as part of fulfillment for the M.D (Branch-IV Microbiology) Degree examination of Tamil Nadu Dr.M.G.R. Medical University, to be held in April 2015

Certificate

This is to certify that the dissertation entitled "Utility of loop-mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis: A pilot study" is a bonafide work done by Dr. Mallika Sengupta towards the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R. Medical University, to be held in April 2015.

Dr. John Antony Jude Prakash	**Dr. V. Balaji**	**The Principal**
Professor and Guide	**Professor and Head**	**Christian Medical College**
Department of Microbiology	**Department of Microbiology**	**Vellore - 632004**
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Declaration

I hereby declare that this M.D. Dissertation titled "Utility of loop-mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis: A pilot study" is a bonafide work done by me under the guidance of Dr. John Antony Jude Prakash, Professor, Department of Microbiology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

Mallika Sengupta

Dr. Mallika Sengupta

Department of Microbiology

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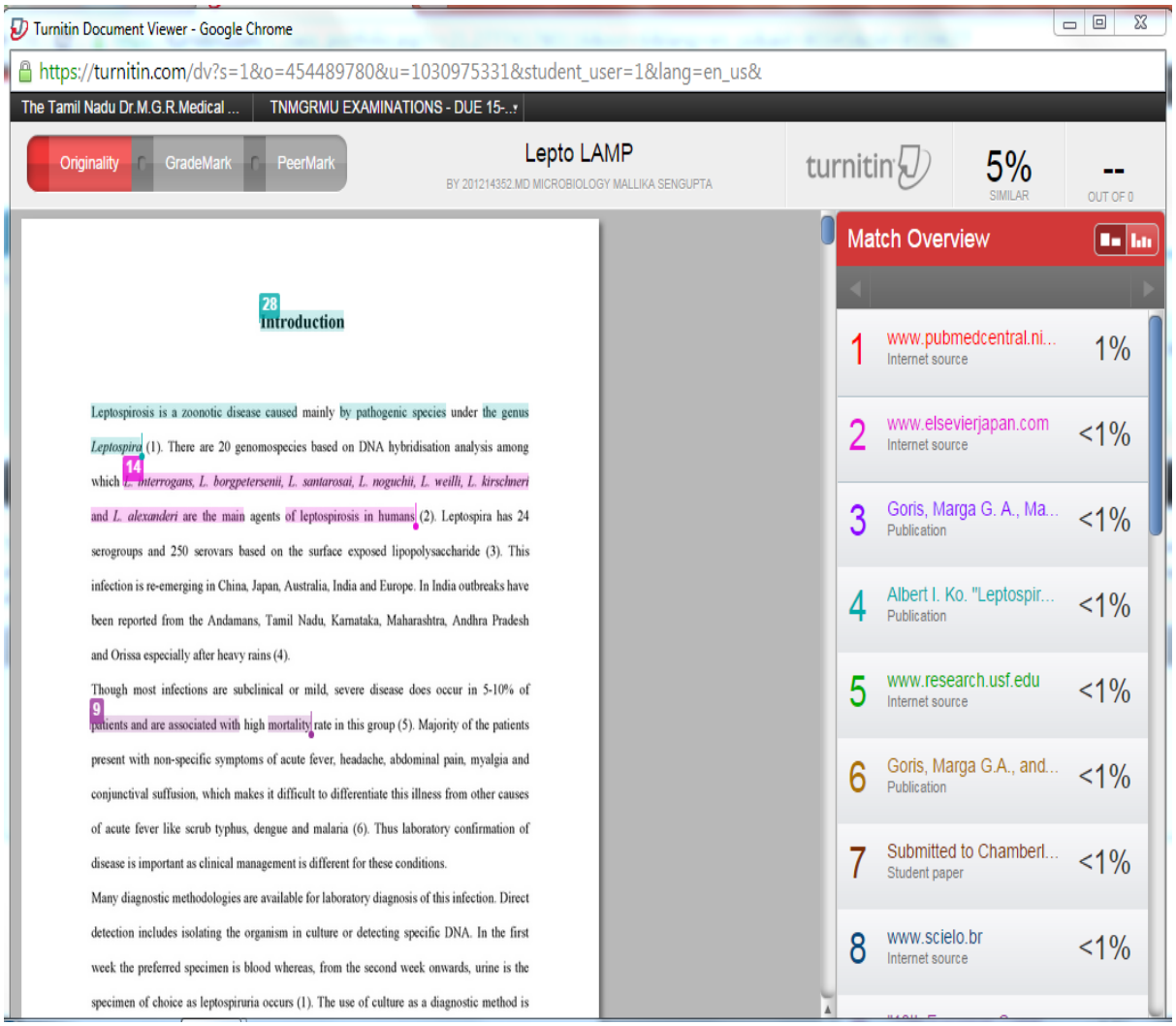
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Introduction
Leptospirosis is a zoonotic disease caused mainly by pathogenic species under the genus Leptospira (1). There are 20 genomospecies based on DNA hybridisation analysis among which L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilli, L. kirschneri and L. alexanderi are the main agents of leptospirosis in humans (2). Leptospira has 24 serogroups and 250 serovars based on the surface exposed lipopolysaccharide (3). This infection is re-emerging in China, Japan, Australia, India and Europe. In India outbreaks have been reported from the Andamans, Tamil Nadu, Karnataka, Maharashtra, Andhra Pradesh and Orissa especially after heavy rains (4).
Though most infections are subclinical or mild, severe disease does occur in 5-10% of patients and are associated with high mortality rate in this group (5). Majority of the patients present with non-specific symptoms of acute fever, headache, abdominal pain, myalgia and conjunctival suffusion, which makes it difficult to differentiate this illness from other causes of acute fever like scrub typhus, dengue and malaria (6). Thus laboratory confirmation of disease is important as clinical management is different for these conditions.
Many diagnostic methodologies are available for laboratory diagnosis of this infection. Direct detection includes isolating the organism in culture or detecting specific DNA. In the first week the preferred specimen is blood whereas, from the second week onwards, urine is the specimen of choice as leptospiruria occurs (1). The use of culture as a diagnostic method is
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Foremost, I want to thank the Almighty for His blessings that enabled me to complete this dissertation.

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A special thanks to all my friends and seniors in the PG Room.

I also thank all the patients who participated in the study without whom this study would not have been possible.

Last but not the least, I thank my family especially my parents and my husband Anirban for their patience, love and support.

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Dear Dr. Mallika Sengupta,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

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Dear Dr. Mallika Sengupta,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Utility of loop-mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis: A pilot study." on December 5, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Patient Information Sheet and Informed Consent Form (English, Tamil, Hindi and Telugu)
3. Proforma
4. Cvs of Drs. Mallika Sengupta,. John Antony Jude Prakash, V. Balaji, Saritha, Dolly Daniel,.K.P. Abhilash, OC Abraham, Thambu David, Sowmya Satyendra.
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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

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A sum of Rs. 40,000 (Rupees Forty Thousand only) will be granted for the first year and consequently for the second year the rest Rs 40.000(Rupees Forty Thousand only) will be granted. Overall Rs. 80,000(Rupees Eighty Thousand only) will be granted for Two Years.

Yours sincerely

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Abbreviations used

AP	Andhra Pradesh
CDC	Centre for Disease Control and Prevention
CSF	Cerebrospinal fluid
DFM/DGM	Dark field microscopy
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbant assay
GM	Geometric mean
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
LAMP	Loop-mediated isothermal amplification assay
LPS	Lipopolysaccharide
MAT	Microscopic agglutination test
MSAT/SAT	Macroscopic slide agglutination test
OD	Optical density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
TMB	Tetramethylbenzidine
TN	Tamil Nadu
WB	West Bengal
WHO	World Health Organisation

Contents

Title of Abstract: Utility of loop-mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis: A pilot study

Department: Department of Microbiology

Name of the candidate: Dr. Mallika Sengupta

Degree and Subject: M.D. (Branch-IV Microbiology)

Name of the Guide: Dr. John Antony Jude Prakash, Professor, Department of Microbiology

Introduction: Leptospirosis is a zoonosis caused by 19 genomospecies of pathogenic *Leptospira* requiring laboratory confirmation. Culture and microscopic agglutination test (MAT) are available only in reference centres, whereas molecular assays like PCR and LAMP are good alternatives. LAMP has been evaluated by only four research groups. Currently, most laboratories use IgM ELISA for diagnosis of leptospirosis.

Objectives: To assess the usefulness of loop-mediated isothermal amplification (LAMP) assay, PCR and ELISA for the diagnosis of leptospirosis after validation of the ELISA.

Materials and Methods: The diagnostic cut off for IgM ELISA was determined using 100 plasma samples, 20 sera from healthy blood donors and serum samples from patients of scrub typhus with eschar (n=50), sepsis (n=20), enteric fever (n=20), dengue (n=17) and malaria (n=17). Three leptospira IgM ELISA kits (PanBio, Virion serion, Inbios) and IgG ELISA (Verion serion) were tested. After obtaining informed consent, 150 serum samples and clinical data were collected from adult patients (≥18 years) with acute undifferentiated fever (≥100º F) of duration ≤ 15 days without eschar, who were malaria and blood culture negative. The serum samples were tested for IgM antibodies to leptospira by PanBio ELISA. PCR for *rrs* gene and LAMP assay for LipL32 and LipL41 were performed on all the samples.

Sequencing was done for 2 samples to assess amplification specificity. These samples were also tested for IgM antibodies to scrub typhus by ELISA (InBios). Convalescent sera could be collected from 32 patients and tested for IgM antibodies to leptospira.

Results: The plasma and serum samples of the healthy blood donors showed similar results by ELISA. The cut-off determined for IgM ELISA by PanBio, InBios, Virion serion was ≥20 PanBio units, ≥1 OD, ≥0.7 OD respectively, whereas it was ≥2.7 OD for Virion serion IgG ELISA. Among the 150 samples tested, three samples were positive by PCR, LAMP and IgM ELISA, two by only PCR, seven only by LAMP and 40 positive by IgM ELISA alone fulfilled modified Faine's criteria. The LAMP assay was found positive in sera from patients with fever ≤7 days. BLAST analysis of the 2 sequenced PCR amplicons confirmed that the amplified DNA was *Leptospira interrogans*. Totally, 56 samples had IgM antibodies to scrub typhus of which 22 had IgM antibodies to both leptospira and scrub typhus. Decreased urine output, platelet count, severe jaundice and renal damage were significantly related to leptospirosis ($p<0.5$). Among the convalescent sera 10 patients continued to have IgM antibodies to leptospira.

Conclusion: LAMP assay is a reliable test for diagnosis of leptospirosis in the first week of illness whereas IgM ELISA forms the mainstay of diagnosis from second week onwards.

Keywords: Leptospira, LAMP, PCR, ELISA, Validation

Introduction

Leptospirosis is a zoonotic disease caused mainly by pathogenic species under the genus *Leptospira* (1). There are 20 genomospecies based on DNA hybridisation analysis among which *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilli, L. kirschneri* and *L. alexanderi* are the main agents of leptospirosis in humans (2). Leptospira has 24 serogroups and 250 serovars based on the surface exposed lipopolysaccharide (3). This infection is re-emerging in China, Japan, Australia, India and Europe. In India outbreaks have been reported from the Andamans, Tamil Nadu, Karnataka, Maharashtra, Andhra Pradesh and Orissa especially after heavy rains (4).

Though most infections are subclinical or mild, severe disease does occur in 5-10% of patients and are associated with high mortality rate in this group (5). Majority of the patients present with non-specific symptoms of acute fever, headache, abdominal pain, myalgia and conjunctival suffusion, which makes it difficult to differentiate this illness from other causes of acute fever like scrub typhus, dengue and malaria (6). Thus laboratory confirmation of disease is important as clinical management is different for these conditions.

Many diagnostic methodologies are available for laboratory diagnosis of this infection. Direct detection includes isolating the organism in culture or detecting specific DNA. In the first week the preferred specimen is blood whereas, from the second week onwards, urine is the specimen of choice as leptospiruria occurs (1). The use of culture as a diagnostic method is limited by its long turn over time, requiring at least 6-8 weeks for growth (4). Dark field microscopy requires the presence of $\geq 10^4$ organisms/ml as does detection of antigen in blood and urine by ELISA or RIA. In addition, dark field microscopy has a low sensitivity (40.2%) and specificity (61.5%) compared to culture (7).

PCR targeting the 16S rRNA has been used to detect the presence of leptospires in serum, urine, CSF and autopsy tissue and has been found to be more sensitive than culture (1). Its value lies in the fact that it can diagnose the disease very early in the first week of illness before the appearance of antibodies and hence helps in early initiation of treatment.

The genes commonly used for detection of leptospiral DNA by PCR are LipL32 coding for outer membrane lipoprotein LipL32 (8), *rrs* for 16S rRNA (9) and *sec*Y coding for pre-protein translocase SecY protein (10). PCR is expensive and needs costly equipments, reagents and technical expertise.

LAMP (loop-mediated isothermal amplification) is an alternative method of DNA amplification with high specificity, efficiency and occurs under isothermal conditions. It employs a DNA polymerase and a set of four specially designed primers that recognize six DNA sequences on the target DNA (11). The final products of LAMP are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops. These loops are formed by annealing between alternately inverted repeats of the target in the same strand. These help in simple, easy, selective detection by electrophoresis (5). The reaction results in the accumulation of huge amount of target and simply requires a laboratory water bath or heating block to maintain a constant temperature of 60–65°C. Detection of products is done by agarose gel electrophoresis, real-time monitoring in an inexpensive turbidometer or in the form of a colour change with an intercalating dye. Moreover, the LAMP assay is not affected by polymerase inhibitors (12).

The utility of LAMP for the rapid and specific diagnosis of leptospirosis has been evaluated by four different groups of researchers. Lin *et al* in 2009 developed a LAMP assay targeting the LipL41 gene and reported the lower detection limit of 100 genome equivalents similar to PCR in mouse kidney sample. The assay was easier and inexpensive compared to PCR (13).

Sonthayanon *et al*, developed another LAMP assay targeting the *rrs* gene and found the analytical sensitivity of 10 genome equivalents (6). They also performed clinical evaluation of the *rrs* LAMP and the LipL41 LAMP in blood samples. The sensitivity and specificity for *rrs* LAMP was 43.6% and 83.5% respectively while for the LipL41 LAMP the sensitivity and specificity was 37.6% and 90.2% respectively (6).

Since leptospiral DNA has been detected from urine sample both in early and late disease by PCR compared to blood, Koizumi *et al* developed a *rrs* LAMP assay which can be used in heated urine samples (14). The lower detection limit of the assay were 2 and 10 genome equivalents per reaction under heat-denaturing and non-denaturing conditions, and the performance of the assay was evaluated in urine of field mice. The assay was positive in 11 of 12 culture positive boiled urine and in 10 of 11 culture positive urine pellet samples. The LAMP assay was also positive in two culture negative samples, suggesting a higher sensitivity than culture. As shedding of the leptospira occurs intermittently, the authors suggested repeated testing using nucleic acid amplification.

Suwancharaoen *et al* evaluated the LAMP assay in 22 pathogenic and 2 non-pathogenic leptospira strains and found the sensitivity was better than PCR and specificity was excellent for pathogenic species of leptospira (15).

MAT (microscopic agglutination test) is the reference method for serological diagnosis of leptospirosis. The presence of leptospiral antibody in the patient's serum is determined by incubating it with serogroup specific live leptospiral antigens and microscopically observing for the presence of agglutination of the antigen (12). A fourfold rise in titre between acute and convalescent sera is diagnostic of the disease. As the baseline titre in endemic population is high, only titres of >1:800 with a compatible disease is indicative of leptospirosis (1). Drawbacks of MAT are its complex procedure and interpretation, time-consuming and

hazardous nature because of the risk of exposure to the live antigen, and high degree of cross reactions between different serogroups and cross-reactions in other unrelated infections like syphilis, viral hepatitis, HIV, relapsing fever, Lyme's disease, legionellosis and autoimmune diseases. It is insensitive particularly, in acute phase and in patients with severe disease who die before seroconversion (1).

Enzyme linked immunosorbant assay (ELISA): IgM antibody detection by ELISA is the most widely used method for diagnosis of leptospirosis. The advantage of ELISA is that it can be performed easily with less infrastructure and technical expertise and is inexpensive compared to MAT. In addition, the ELISA can be automated, the result is objective, especially once a diagnostic cut-off has been decided upon, therefore having less interobserver/intraobserver variation.

The ELISA for detection of antibodies in leptospirosis should be validated. The usage of well characterized archived specimens, consisting of those with disease and other clinically similar diseases allows rapid assessment of proof of concept and validation of the new assay (16). Assessment of specificity of a particular assay also requires testing with sera diagnosed as other illnesses, which present with the same clinical features.

Aim of the study

To assess the usefulness of a loop-mediated isothermal amplification (LAMP) assay and ELISA for the diagnosis of leptospirosis

Objectives of the study

1. To revalidate the diagnostic cut-off of ELISA used for confirming a clinical diagnosis of leptospirosis.
2. To establish a LAMP assay for detection of leptospirosis.
3. To assess the utility of LAMP assay for diagnosis of leptospirosis.
4. To compare the utility of LAMP, PCR and ELISA for diagnosis of leptospirosis.
5. To correlate clinical features with the diagnosis of leptospirosis.

Review of Literature

History

Leptospirosis is a zoonosis which dates back to time immemorial. In ancient China, leptospirosis was known as an occupational hazard for the rice farmers and the Japanese name 'akiyami' which means autumn fever is still prevalent today (17). Several cases of leptospirosis were mentioned but the actual description of the disease or the causative agent was not known for many years. In 1886 a German physician Adolf Weil, Professor of Medicine at Heidelberg first described the disease leptospirosis. It is in his honour that the severe form of leptospirosis is named as Weil's disease. In 1907 Arthur Stimson first visualised the organism in silver stained preparations of liver tissue of a patient who died of supposed yellow fever, the causative agent of which was not known. Later, it was diagnosed that the patient had Weil's disease. At that time, it was only seen under the microscope but not grown in culture media (4). It was first cultured in 1915 by Inada *et al* in Japan, from blood of coal miners with infectious jaundice, who named the causative agent as *Spirochaeta icterohaemorrhagiae* (18) and Uhlenhuth and Fromme in Germany, who isolated it from soldiers and named it as *Spirochaeta icterogenes*, after inoculation of blood into guineapigs (19). In 1914, a saprophytic leptospira was isolated from fresh water. It was called *Leptospira biflexa* (4). The name Leptospira meaning "thin spirals" was given by Hideyo Noguchi in 1918 (20). In 1917, rat was identified as the source for transmission of the disease to humans (21). Between 1920 and 1950, different serotypes with their geographic distribution, and hosts were identified (22). During 1960s, the detailed structure of leptospira was seen under the electron microscope. In 1966, Yanagawa and Faine conclusively showed that leptospira are similar to other bacteria in structure. They have a characteristic antigen on their cell surface (4).

Agent

The causative agent of leptospirosis is leptospira. Leptospira belongs to the phylum *Spirochaetes*, order *Spirochaetales* and family *Leptospiraceae* (23). It is a thin spiral bacteria measuring 0.1µm by 6-20µm (24). The name is derived from the Greek word "leptos" meaning thin and Latin word "spira" meaning coil (24). They are very slender, delicate organisms. They have pointed ends which are bent in hooks, sometimes the ends may not have a hook. The straight forms of leptospira travel much slower than the hooked forms. Leptospira exhibit both translational and rotational motility or cork-screw motility because of the presence of periplasmic axial filamentous flagella (1).

Traditionally, leptospira was classified into two species - the pathogenic leptospira which are pathogenic or parasitic in humans and animals known as *Leptospira interrogans* and the non-pathogenic free-living saprophytic species called *Leptospira biflexa*. Both the species have many serogroups in them (25). Based on DNA hybridization analysis leptospira are now classified into several genomospecies (3). On the basis of the surface exposed liopopolysaccharide, leptospira are classified into various serogroups and each serogroup has numerous serovars in it. Currently there are 24 serogroups and 250 serovars (3) and 20 genomospecies of leptospira (2). The pathogenic genomospecies which are predominantly responsible for causing human diseases are *Leptospira interrogans, Leptospira kirschneri, Leptospira borgpetersenii, Leptospira noguchii, Leptospira alexanderi, Leptospira weilii, Leptospira santarosai* (26). The genomospecies and the serogroups are not mutually exclusive – one serogroup may be present in more than one genomospecies (27).

Table 3.1 shows the different genomospecies along with the serogroup and number of serovars included in it.

Table 3.1: Genomospecies and serogroups of leptospira (26,27)

Genomospecies	**Serogroup**	**Serovar**
L. interrogans	Icterohaemorrhagiae	18
	Australis	9
	Autumnalis	8
	Bataviae	5
	Canicola	12
	Pomona	8
	Grippotyphosa	3
	Sejroe	10
L. kirschneri	Autumnalis	5
	Canicola	3
	Pomona	3
	Grippotyphosa	7
L. borgpetersenii	Sejroe	10
	Javanica	10
L. noguchii	Icterohaemorrhagiae	5
	Australis	4
	Bataviae	2
L. alexanderi	Javanica	1
L. weilii	Javanica	3
	Sarmin	1
L. santarosai	Bataviae	5
	Pomona	2
	Sejroe	5

Cell wall structure

Leptospira has an inner membrane (IM) and an outer membrane (OM). The peptidoglycan cell wall is associated with the inner membrane, iron transporter (FeoAB), penicillin-binding proteins (PBPs) and the lipoprotein LipL31. The leptospiral outer membrane contains lipopolysaccharide (LPS), the transmembrane porin, outer membrane protein L1 (OmpL1) and the lipoproteins LipL32, LipL36 (on the inner surface of the OM), LipL41 and LigB (28) as shown in the Figure 3.1 below.

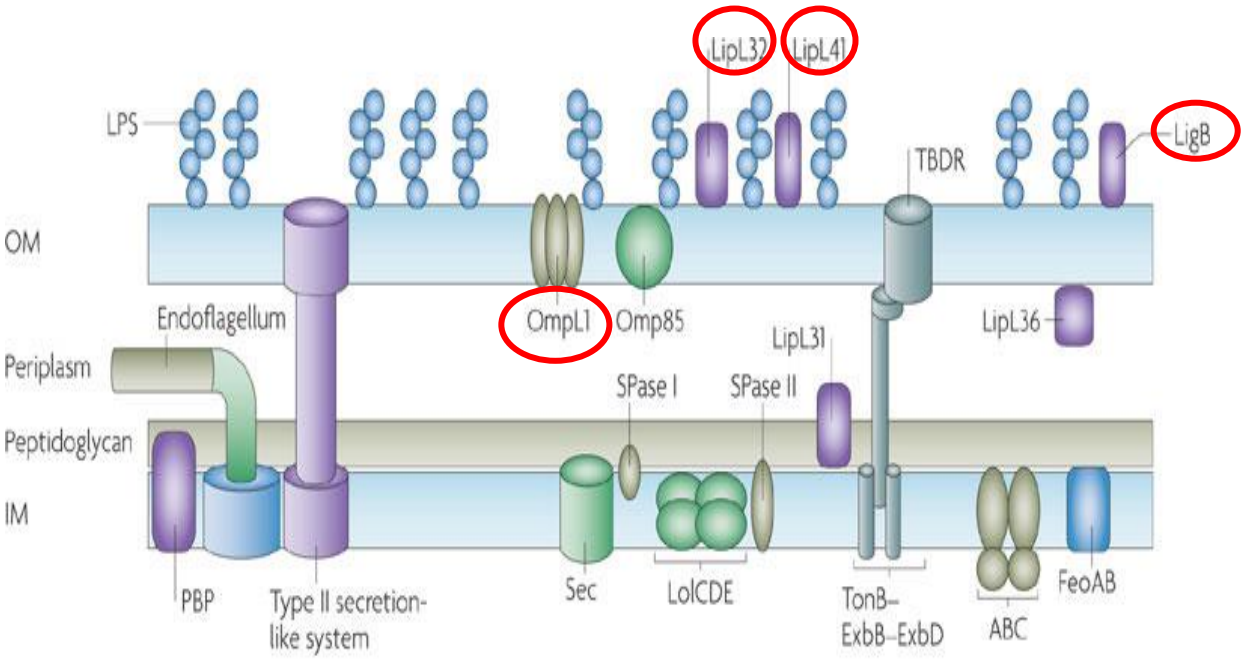


Figure 3.1: Cell wall structure of leptospira Reference (28)

These outer membrane proteins like LipL32, LipL41, OMPL1 and LigB are used as antigens for serological tests as well as targets for different molecular assays. In a study by Haake *et al*, it shows that these targets have high specificity for *Leptospira interrogans* (29). Hence these targets are used for detection of pathogenic leptospira.

Epidemiology

Global Epidemiology

There annual incidence of leptospirosis is 5 per 100,000 population worldwide. The median annual incidence varies in the different continents.

According to the second meeting on leptospirosis by World Health Organisation held in 2010 (30) the incidence of leptospirosis is shown in table 3.2.

Table 3.2: Global incidence of leptospirosis

Region	Incidence
African Region	95.5 per 100,000 population
Western Pacific	66.4 per 100,000 population
The Americas	12.5 per 100,000 population
South-East Asia	4.8 per 100,000 population
Europe	0.5 per 100,000 population

The annual incidence of leptospirosis varies in different parts of the world. In a study done by Pappas G *et al* the annual incidence in United Kingdom and United States of America is as low as 0.6 and 0.1 per million population respectively (31). In another study, done by Traxler *et al*, in USA, between 1998 to 2009 the average annual rate of hospitalisations with leptospirosis is 0.6 per million population (32).

In Asia, the incidence is high in Sri Lanka (54 per million population) and Thailand (48.9 per million population) (31) while it is low in Singapore (2 per million population). In India, although the actual incidence of leptospirosis is not known, it is considered as probably endemic for leptospirosis (31).

Leptospirosis in India

In India, the overall annual incidence is not known. According to the data available, the annual incidence is highest in Andaman Islands with 500 per million population (31). In a multicentric study done by Indian Council of Medical Research the disease burden was found to be 12.7% among 3682 patients of acute febrile illness in 13 different centers (33). The prevalence of leptospirosis is dependent on the geographical area, the number of patients recruited and the method of diagnosis.

The varying prevalence of leptospirosis in febrile patients in different parts of the country is shown in table 3.3.

Table 3.3: Indian studies on leptospirosis prevalence

Year	Place	Population	Number tested	Prevalence	Method of diagnosis	Reference
2002–2008	Kolkata	Fever patients	404	52.97%	IgM ELISA	(34)
2004–2008	Chandigarh	Fever patients	1391	16.67%	IgM ELISA	(35)
2000–2010	Delhi	Fever patients	1453	26.9%	IgM ELISA	(36)
2006	Andaman Islands	High risk population	611	52.7%	MAT	(37)

In Chandigarh, Sethi *et al* showed that the incidence of leptospirosis had increased from 11.7% in 2004 to 20.5% in 2008.

Leptospirosis in Tamil Nadu and Pondicherry

In Tamil Nadu, the prevalence of leptospirosis is variable depending upon the population recruited. The prevalence is very high in tribal populations (38) as shown in table 3.4.

Table 3.4: Data on leptospirosis from Tamil Nadu and Pondicherry

Year	Place	Population	Number tested	Prevalence	Method of diagnosis	Reference
1995	Pondicherry	Febrile jaundiced patients	_	12%	MAT	(4)
2005-2006	Pondicherry	Patients with fever	110	60%	MAT IgM ELISA	(39)
2007-2008	Vellore	Patients with fever	398	3%	IgM ELISA	(40)
2008	Marakkanam village	Irula tribal population	72	61.1%	MSAT	(38)
2008	Chennai	Urban patients with fever	3830	19.5%	MAT DFM	(41)
2008-2010	Vellore district	Patients with fever	129	7.75%	MSAT MAT	(42)
2011-2012	Villupuram district	Urban patients with fever	1502	4.32%	IgM ELISA MSAT	(43)
2011- July 2014	Vellore	Fever patients	1833	3.49%	IgM ELISA	Not published

Leptospirosis is a zoonosis due to an interaction of host factors, agent factors and environmental factors.

Host Factors

There are many animals that commonly develop or spread leptospirosis as reservoirs which include rodents, raccoons, cattle, swine, dogs, horses, buffaloes, sheep, goats among others (44). The animals once infected may remain as carriers throughout their life and shed the bacilli forever. The soil and vegetation are contaminated with the urine of these chronic carrier animals. The major route of transmission to humans is by contact with infected animals, soil or water where the bacteria are present (24). The risk is more in farmers, sewer workers, slaughterhouse workers, veterinarians and animal caretakers, fresh water fishermen, dairy farmers, military personnel (45). A study by Sugunan A P *et al* showed that barefoot walking and standing in water while working are risk factors associated with acquiring the infection (46). Earlier it was thought that leptospirosis is a disease of the poor and people of lower socioeconomic status. But now, the concept has changed. It can also occur among those involved in outdoor freshwater activities like swimming, rafting, kayaking etc. There is a report in Sri Lanka of 20 people who went for rafting and 17 of them developed antibodies to leptospira as shown by MAT or IgM ELISA (47). Sometimes, leptospirosis is acquired in the course of travel. Lagi F *et al* reported that two Australian tourists were infected with leptospira after immersion in a canal in Venice in Italy (48). In a study done on 60 patients with leptospirosis by Guerrier P *et al* it was shown that leptospirosis is more severe in adolescents than in children (49). Another study by Spichler A *et al* showed that among 370 patients, adults had higher rates of hepatic and renal derangement as shown by jaundice, oliguria, raised bilirubin and creatinine levels in comparison to the children. The overall case fatality rate was 27% for adults and 5% for children (50).

Agent factors

The severity of the illness is also dependent on the infecting serovar. The common species causing severe disease in humans are *L. interrogans, L. kirschneri, L. noguchii, L. santarosai, L. borgpetersenii, L. weilii, L. alexanderi, L. fainei* and *L. meyeri.* There are some species like *L. inadai, L. broomii* and *L. licerasiae* of indeterminate pathogenicity in causing disease and others like *L. wolbachii* and *L. biflexa* which are non - pathogenic (24).

Environmental Factors

The risk factors associated are –

i. Exposure to animals: Leptospirosis is a zoonosis, classically transmitted by contact with infected rat urine. The animals serve as renal carriers of leptospira (51). In a study in Chile, it was shown that presence of dogs and rodents were related more with the water samples of puddles which had leptospira as detected by PCR (52).

ii. Poor sanitation and inadequate waste disposal: The disease is more common in urban slums. In a study, it showed that slum residents had a higher risk of greater than 3% per year of acquiring leptospira infection (53). Garbage is a breeding place for rodents. It is associated with construction and land – use in the megacities, during fairs and festivals when there is more movement of people and less maintenance of hygiene (54). In the cities the drains get blocked and water overflows onto the streets resulting in people coming in contact with wet mud (55).

iii. Temperature: Leptospirosis occurs during the hot months of the year as the organism can survive for long periods in the hot months (54).

iv. Rainfall and flooding: Leptospirosis occurs more during the monsoon and is more common in low-lying areas. This is probably due to contamination occurring during floods (54). The major outbreaks of leptospirosis in India have occurred during the rainy

season. Table 3.5 shows the data with the corresponding methods for detection of the disease. Currently no published data is available on leptospirosis outbreaks after 2006.

Table 3.5: Outbreaks of leptospirosis in India (1999 – 2006)

Year	Place	Number of Cases	Positives	Methods of Diagnosis	Reference
1999 (October-November)	Orissa	142	28.17% 19.71%	IgM ELISA MAT	(56)
2000 July	Mumbai	102	36.27%	IgM ELISA DGM	(57)
2000 (July- September)	Mumbai	53	33.9%	IgM ELISA	(58)
2002 (October-November)	Andaman Islands	156	33.33%	MAT	(46)
2002 (July –October)	Kerala	340	282 255	MAT IgM ELISA	(59)
2003	Chennai	69	35%	MAT PCR	(60)
2003	Raichur	6 villages	1516	IgM ELISA	(61)
2005 (July- September)	Mumbai	942	34.3%	Leptotek, Dridot, Leptocheck	(62)
2005 (August- October)	Chittoor	86	56.97%	DGM IgM ELISA	(63)
2006	Gujarat	1258	55.8%	MAT, PCR IgM ELISA	(64)

Figure 3.2: Leptospirosis epidemiology in India

Pathogenesis

Leptospires enter the body through cuts, abrasions or scratches on the skin, mucous membranes and conjunctivae and rarely through inhalation of droplets. Upon entry, hematogenous dissemination, penetration of tissue barriers and transendothelial migration of the organisms help in the spread. Severe vascular injury causes pulmonary haemorrhage, tubular epithelial cell necrosis and ischaemia in renal cortex and liver damage. The virulence factors are toxin production, adhesins, and other surface proteins (24).

Clinical features

Leptospirosis has a wide spectrum of disease ranging from subclinical illness to severe disease. A self – limiting illness is seen in 90% cases. The mean incubation period is 10 days (range of 5 – 14 days). In classical leptospirosis, there are two phases of the disease – a septicaemic phase followed by an immune phase (24).

The illness begins with a high fever of 38 – 40^0 C associated with headache, chill, rigor and myalgia. In the septicaemic phase, the other symptoms are abdominal pain, anorexia, nausea, vomiting, diarrhoea, cough and pharyngitis. Conjunctival suffusion and tenderness in calf muscles and lumbar areas are characteristic physical findings though these are present in a minority of cases. A maculopapular rash occurs rarely. On examination, lymphadenopathy, splenomegaly and hepatomegaly may be present. This acute phase lasts for 5 – 7 days (24). As the symptoms are non-specific, leptospirosis should be differentiated from malaria, dengue and rickettsial fever (65).

In the immune phase the organism is cleared from blood. It can be detected in tissue and urine. In addition to the acute phase symptoms, in the immune phase, the characteristic symptoms are jaundice, renal failure, cardiac arrhythmias, pulmonary symptoms, aseptic meningitis, conjunctival suffusion with or without haemorrhage and photophobia (24).

In a study done in India, the common clinical features in leptospirosis patients are fever (100%), myalgia (78.4%), headache (41.2%), oliguria (29.4%), respiratory distress (25.5%) and bleeding manifestations (9.8%). Icterus was found in 74.5% of patients and tachypnoea in 52.9% (66).

In the severe form of the disease, which occurs in 10% patients, the illness is complicated with combination of renal failure, liver failure and pneumonitis with haemorrhagic diathesis and aseptic meningitis. The severe form of leptospirosis or Weil's disease is a triad affecting liver, kidney and lung (Figure 3.3). The mortality ranges from 5 - 40% in the severe disease (24).

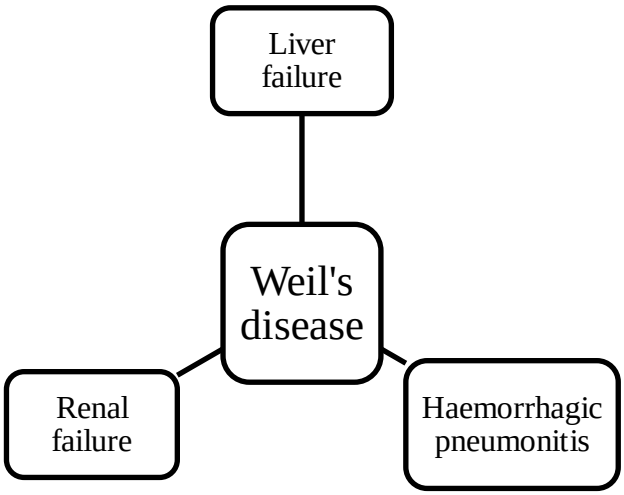


Figure 3.3: Weil's disease triad

Co – infection

Leptospira infection can occur in patients with other diseases. Concurrent infection of leptospirosis can occur with typhoid fever, brucellosis and rickettsial infection (67). Simultaneous detection of antibodies to leptospira and scrub typhus is quite common. In 2003, a case of concurrent infection of scrub typhus and leptospira was reported in a patient with acalculous cholecystitis (68). In 2012, another case of co-infection with scrub typhus

and leptospira was reported by Wei *et al*. However, both the cases responded well to management (69). A similar case was reported from the Himalayan region in India in 2012 (70). In 2013, a study by Sonthayanon *et al* showed that out of the 82 cases who had serological evidence of dual infection of leptospira and scrub typhus, 5(6%) had molecular evidence of combined infection (71). Among the other diseases, two cases of malaria and leptospirosis were reported in 2011 (72). Malaria, leptospirosis and dengue occurred together in 0.1% while dengue and leptospirosis were seen in 1.6% patients with acute febrile illness in Jamaica in 2013 (73). Dengue and leptospira combined infection was also seen in Puerto Rico in 2010 (74). In 2012, four cases were reported with co-infection of leptospira and *Burkholderia pseudomallei* in Malayasia (75).

Leptospira can be isolated from blood, urine and CSF (Figure 3.4).

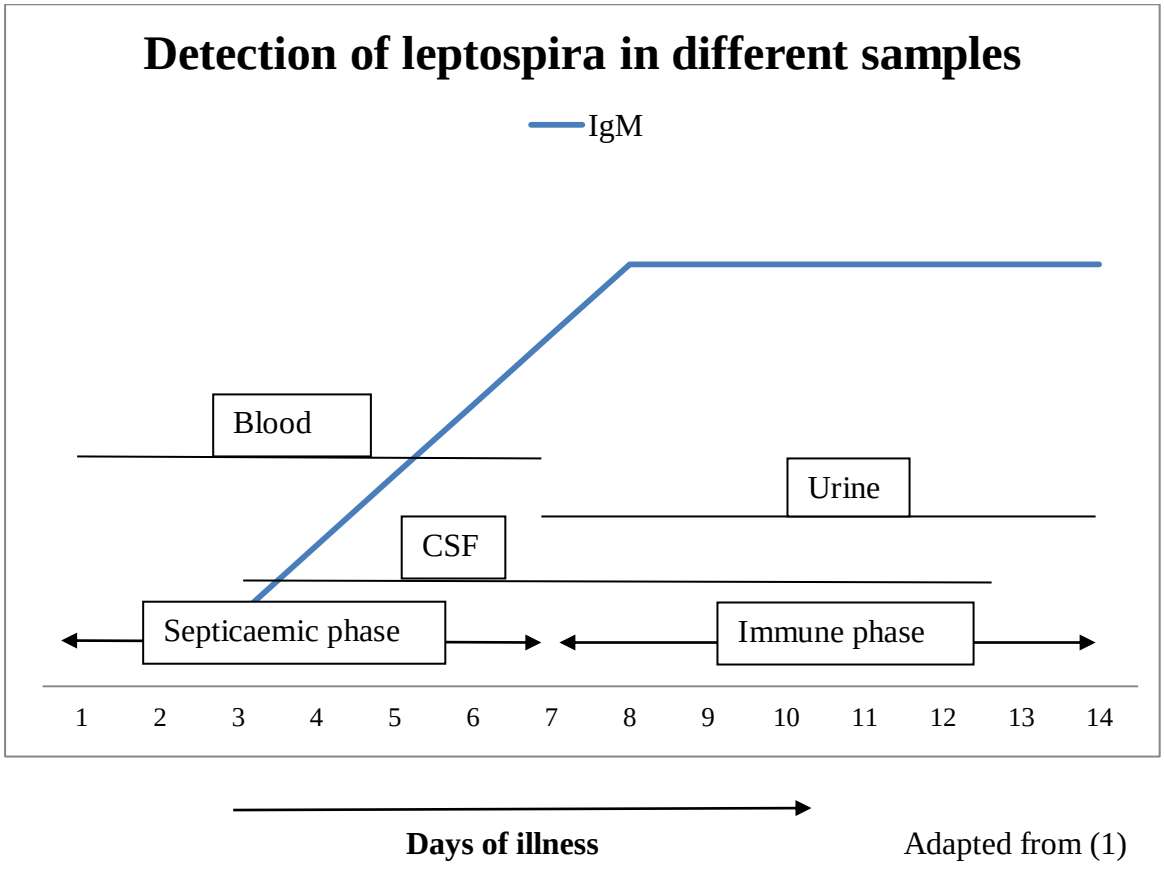


Figure 3.4: Samples used for diagnosis of leptospirosis

Laboratory Diagnosis

The common methods used for diagnosis of leptospirosis are given below (Figure 3.5)

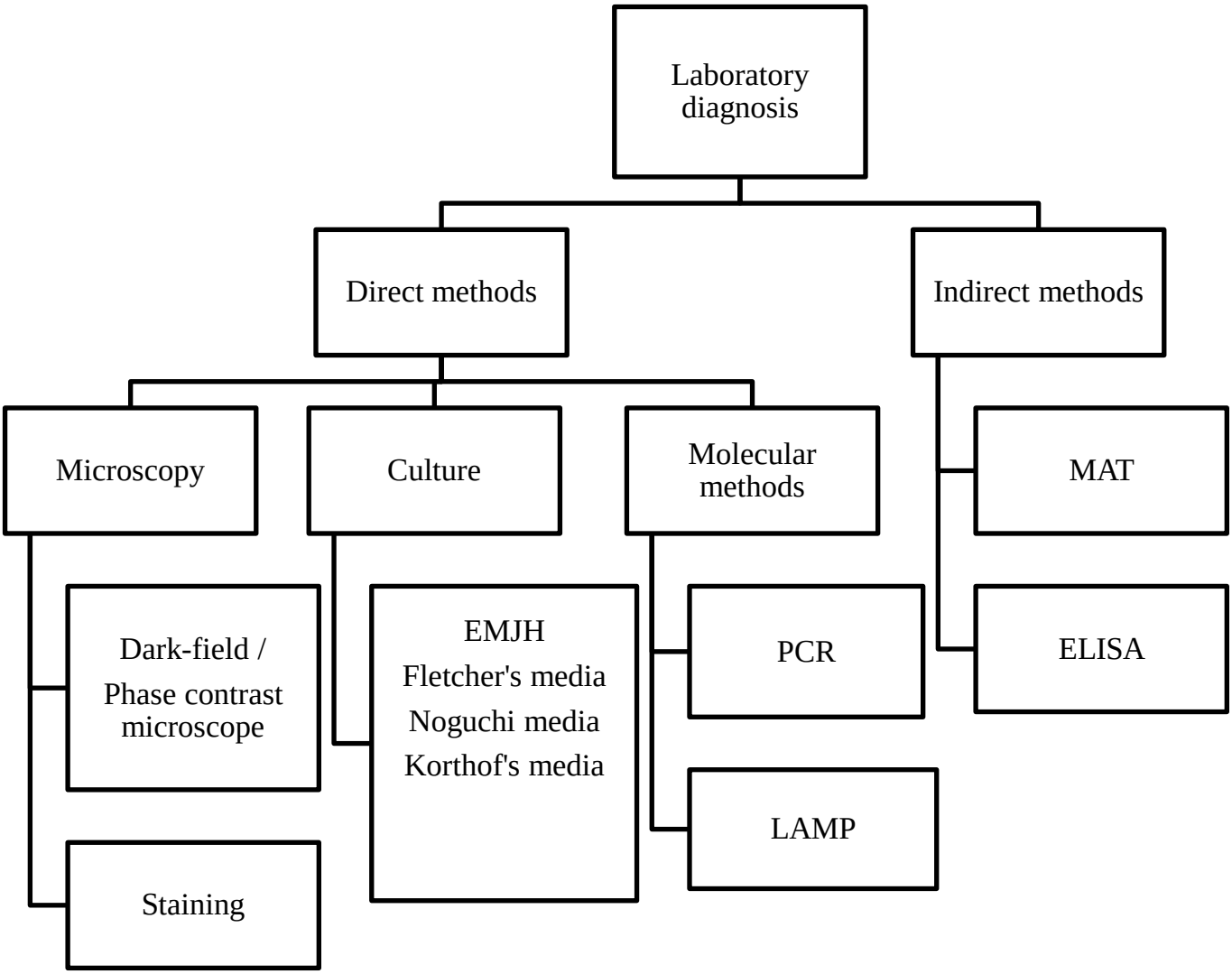


Figure 3.5: Flowchart of commonly used methods for diagnosis of leptospirosis

Microscopy

Dark field/ phase contrast microscope

As leptospira is a thin, delicate organism, it cannot be seen under the light microscope. It is visible only under dark field microscope or phase contrast microscope. It has a characteristic cork – screw motility due to a combination of translational and rotational movements (23). The limit of detection of dark field microscopy is 10^4 leptospires/ml (1). A buffy coat method has a lower detection limit of 10^3 leptospires/ml (76). A better method is microscopic examination of double centrifuged anticoagulated blood. It can be used for direct microscopy in blood, urine, dialysate fluid and for confirmation of culture. Microscopy of blood is useful only during the first few days of the acute illness during the phase of leptospiremia. However, it can sometimes be detected as early as the fourth day before the appearance of symptoms. Dark field microscopy is a rapid method for diagnosis of leptospirosis (1). Microscopy of urine should be performed immediately as the acidic urine causes lysis of the organism. The organism is shed intermittently in urine giving false negative results (77). False positives can occur with artefacts like RBCs, fibrils and protein threads (1).

In a study by Sharma *et al*, 297 cases of clinically suspected leptospirosis were recruited and tested by dark field microscopy and IgM ELISA for antibody detection. The sensitivity and specificity of dark field microscopy was found to be 60% and 61% respectively (78). In another study, it was found that dark field microscopy has a low sensitivity (40.2%) and specificity (61.5%) compared to culture (7).

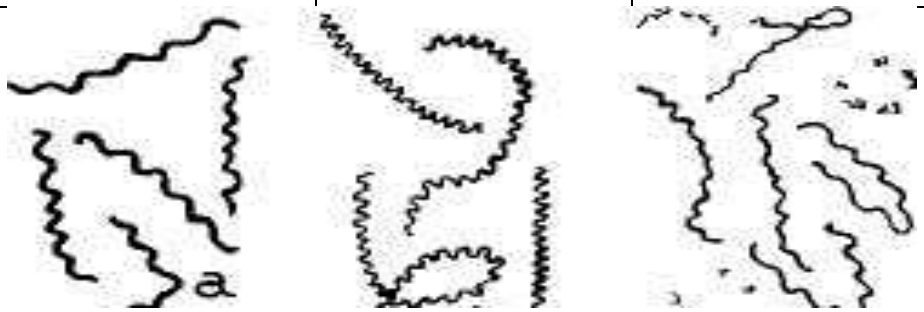
Chandrasekaran S *et al* found that dark field microscopy had a sensitivity of 93.3% in suspected cases of leptospirosis. However, the performance of dark field microscopy deteriorates in samples collected after the first week of illness though it performs very well in the first week of fever (79).

Staining

As the organism is slender and delicate, it cannot be visualized with Gram stain and special staining is required. Immunofluorescence, immunohistochemistry and immunoperoxidase staining are widely used for diagnosis of leptospira in veterinary specimen (80).

Different histopathological stains like Warthin-Starry, Faine, or Dieterle methods are used for detection of leptospira in tissue specimen. Warthin-Starry stain and modified Fontana's stain which are modifications of silver staining are most widely used. In silver staining, they look as brown or black spiral organisms in yellow tissue background and should be differentiated from treponema and borrelia (1). The differences between the three are shown in table 3.6. Other structures like erythrocytes, other bacteria and yeast stain dark, but the characteristic shape and hooked ends of the leptospira differentiate it from other cells (81).

Table 3.6: Differentiating features between leptospira, treponema and borrelia in silver staining

Characteristics	Treponema	Leptospira	Borrelia
Length	5-15µ	6-20 µ	10-30 µ
Width	0.2 µ	0.1-0.3 µ	0.3-0.7 µ
Primary coil	Closely wound	Very closely wound	Loosely wound
Winding	Regular	Regular	Irregular
Schematic diagram			

Culture

Culture is the gold standard for diagnosis of leptospirosis. Leptospira is an obligate aerobe which requires vitamin B1, B12 and long chain fatty acids for its growth. It is incubated at 28 – 30^0C and kept upto 13 weeks before declaring it as negative with regular subculture at 10 – 14 days (1). The different culture media used are given below in table 3.7.

Table 3.7: Culture media used for leptospira

Media	Type	Content	Comments
EMJH media	Enriched Liquid	Albumin, polysorbate80,Na_2HPO_4, NaH_2PO_4, NaCl, NH_4Cl, thiamine	Most widely used
Fletcher's media	Enriched Semisolid	Peptone, beef extract, NaCl, agar, 5-Fluorouracil	Growth seen as Dinger's ring
Korthoff's media	Enriched Liquid	Sodium citrate, Na_2HPO_4, NaH_2PO_4, NaCl, NH_4Cl , peptone, vitamin	
Noguchi media	Enriched Semisolid	Serum, agar, minerals	

The most widely used media for culture of leptospira is Ellinghausen-McCullough-Johnson-Harris (EMJH) media containing oleic acid and albumin. Blood is inoculated at bedside in the media for isolation of leptospira in the first week of illness. The other samples in first week are CSF and dialysate fluid. Multiple blood cultures are required for better yield. In the second week of the illness, urine is a better sample for isolation of leptospira though there is intermittent shedding of the organism. However, the urine should be processed immediately as leptospira is sensitive to acidic environment and antibodies present in urine may lyse the

fragile organism (1). The yield of leptospira is highest (81.3%) in whole blood as compared to surface plasma, deposit from spun plasma and clotted blood (82). In semisolid media the growth of leptospira is seen as a ring below the surface in the media. This is known as Dinger's ring or disk and it goes on increasing with further incubation. The growth is related to oxygen tension in the media (1). The advantage with culture is that it is 100% specific. The disadvantage is that leptospira is difficult to culture and has to be incubated for long period which leads to contamination of the culture media. Serum rich media are required for culture. It has a low sensitivity of 10.5%. Contaminated cultures may be passed through 0.2mm or 0.45mm filter for purification before subculture into fresh medium. The cultured isolates are confirmed by serological methods or molecular methods (1).

Antigen detection methods

Antigen is detected in blood and urine. Several methods like radioimmunoassay (RIA), ELISA, countercurrent immunoelectrophoresis and staphylococcal coagglutination have been tried for antigen detection in blood and urine. RIA has a limit of detection of 10^4 to 10^5 leptospires/ml whereas ELISA needs the presence 10^5 leptospires/ml. An immunomagnetic antigen capture combined with fluoroimmunoassay has shown a detection limit of 10^2 leptospires/ml (1). In a study done by Saengjaruk P *et al*, the antigen detection in urine by monoclonal antigen based ELISA in urine was 75% and 100% on the first day and the fourteenth day of illness (83). In another study done by Nizamuddin M *et al*, 19.2% patients had leptospira antigen detectable in blood by ELISA (84).

Animal inoculation methods

Different animals like guinea pigs, hamsters, gerbils, young rabbits, swiss albino mice and 1 to 3 days old chicks are inoculated intraperitoneally for isolation of leptospira. A drop of peritoneal fluid can be examined with dark-field microscopy for active leptospires from the 3rd to the 7th day (4).

Serology

Microscopic agglutination test

MAT (microscopic agglutination test) is the reference method for serological diagnosis of leptospirosis. The presence of leptospiral antibody in the patient's serum is determined by incubating it with serogroup specific live leptospiral antigens and microscopically observing for the presence of agglutination of the antigen (12). After incubating for about 2 hours at 30°C, the results are taken under a dark field microscope. The last dilution in which ≥ 50% of the leptospires are agglutinated is the titre. Seroconversion or four fold titre rise in paired sera is consistent with the diagnosis of leptospirosis. The significance of a titre in a single sample depends on the frequency of residual titre due to past infections and cross-reacting other diseases in the population (85). The drawbacks of MAT are -

i. It is complex, time-consuming procedure and interpretation is difficult.
ii. Hazardous nature because of the risk of exposure to the live antigen.
iii. Difficulty in maintaining the serovars necessitates continuous weekly subculturing of the strains.
iv. It requires periodic verification of the strains.
v. It gives false negative reactions in delayed seroconversion, which sometimes occurs by 30 days after infection.
vi. High degree of cross reactions between different serogroups and cross-reactions in other unrelated infections like syphilis, viral hepatitis, HIV, relapsing fever, Lyme's disease, legionellosis and autoimmune diseases are due to the persistence of IgM antibodies for long time (24).
vii. It is insensitive particularly, in acute phase and patients with severe disease who die before seroconversion (1).

ELISA

Enzyme linked immunosorbant assay (ELISA): IgM ELISA, IgM capture ELISA and IgM dot ELISA-dipstick to detect IgM antibodies against leptospires have been developed for diagnosis. Different antigens have been used for detection of antibody against leptospira as shown in table 3.8 below.

Table 3.8: Antigens used for detection of IgM antibodies to leptospira by ELISA

Antigen	Compared to	sensitivity	Specificity	Reference
Recombinant Lsa63 produced in E.coli	MAT	93.8%	81.29 %	(86)
Leptospiral Ig-like protein B (LigB)		96.9%	91.08%	(87)
C-terminal coding sequence of ligA (ligA-C) cloned produced in Escherichia coli		92.1%	97.7%	(88)
Purified recombinant antigens rLipL32, rLipL41 and rLigA-Rep (leptospiral immunoglobulin -like A repeat region)		90%	-	(89)
LipL32		96.4%	90.4%	(90)
Recombinant LipL32 antigen		100%	85.1%	(91)
Leptospira fainei (whole cell extract)		94%	99%	(92)
rLipL32/1-LipL21-OmpL1/2-IgM-ELISA		97.5%	-	(93)

An IgM antibody capture enzyme-linked immunoassay using the M20 strain of *Leptospira interrogans* serovar Copenhageni has a sensitivity and specificity of 92.3% and 56.0%

respectively (94). The commercially available ELISA mostly uses the recombinant leptospira antigen for detection of antibodies.

IgM detection is more sensitive than MAT in early disease. The advantage of ELISA is that it can be performed easily with less infrastructure and technical expertise and is inexpensive compared to MAT. In addition, the ELISA can be automated, the result is objective, specially once a diagnostic cut-off has been decided upon, therefore having less interobserver/intraobserver variation. Since many other diseases have similar clinical manifestations the ELISA should be validated. The usage of well characterized archived specimens, consisting of those with disease and other clinically similar diseases allows rapid assessment of proof of concept and validation of the new assay (16).

IgM ELISA is the most widely used method for diagnosis of leptospirosis. Hence, the ELISA should be validated and the cut–off should be determined in the different regions based on the local prevalence of the disease in that particular region.

Rapid tests

Rapid tests like immunochromatographic test, flow-through assay and latex agglutination tests have variable sensitivity and specificity. A simple latex agglutination assay 'Leptorapide' ((Linnodee Ltd, Northern Ireland) was evaluated and had a sensitivity and specificity values of 97·1% and 94·0%, respectively, when compared to the microscopic agglutination test. The agglutination is graded in a score of 5 (95). In another study by Goris *et al* three different rapid tests, LeptoTek Dri Dot (bioMe´rieux B.V. Boxtel, the Netherlands), LeptoTek Lateral Flow (Organon Teknika B.V. Boxtel, the Netherlands) and Leptocheck-WB (Zephyr Biomedicals, Verna Goa, India) were evaluated. Lepto-Tek Lateral Flow and Leptocheck-WB are lateral flow immunochromatographic tests, while LeptoTek Dri Dot is a latex agglutination assay. The overall sensitivity and specificity for the LeptoTek

Dri Dot was 75% and 96%, for the LeptoTek Lateral Flow 78% and 95%, and for the Leptocheck-WB 78% and 98% respectively (96). A novel vertical flow immunochromatography rapid diagnostic test showed a sensitivity of 89.8% and a specificity of 93.7% (97). In a study by Senthilkumar *et al* using recombinant LipL41 antigen for detection of antibody against leptospira, two rapid tests – latex agglutination test and flow-through assay were evaluated. The latex agglutination test had a sensitivity and specificity of 98.7% and 90.45% and the flow-through assay had sensitivity and specificity of 89.09% and 77.70% respectively compared to MAT (98).

The advantages of these rapid tests are that they do not require any training, are easy to perform and can be performed in the resource – limited and field settings. They give results within minutes to four hours (99).

The disadvantage is that they give false positive results with HIV, Hantavirus, Toxoplasma, Lyme disease, malaria, meningococcal meningitis and hepatitis A infection (1).

Other serological tests

A macroscopic slide agglutination test using 12 serovars was used for antibody detection but it had false negative results in the endemic regions (1). A modified macroscopic slide agglutination test with serovar patoc as the antigen has been used for the detection of antibodies to leptospira. Brandao *et al* found that the sensitivity and specificity of macroscopic slide agglutination test was similar to IgM ELISA for detection of antibodies in leptospira infection (100).

Other tests like indirect haemagglutination test using sensitised red blood cells developed by CDC, microcapsule agglutination test using a synthetic polymer in place of red blood cells, counter immunoelectrophoresis and thin-layer immunoassay have been described and evaluated but are not widely used for diagnosis of leptospirosis (1).

Molecular methods

PCR

Polymerase chain reaction (PCR) is one of the most widely used methods for diagnosis of leptospirosis in acute febrile illness. The genes commonly used for detection of leptospiral DNA detection by PCR are LipL32 coding for outer membrane lipoprotein LipL32 (8), *rrs* for 16S rRNA (9) and *sec*Y coding for pre-protein translocase SecY protein (10). PCR has been done for different targets by different groups which are given in the table 3.9 below with their sensitivity and specificity.

Table 3.9: PCR performance for diagnosis of leptospirosis

Year	Sample	Target	Number tested	Compared to	Sensitivity	Specificity	Reference
2006	Blood	16S rRNA	60	MAT Culture	62-72.7%	100%	(101)
2009	Blood	secY gene	133	Culture	89-100%	93-100%	(10)
2010	Blood	*rrs* gene LipL32	266	Culture MAT	56% 43%	90% 93%	(8)
2011	Blood	*rrs* gene	418	Culture	94.8%	-	(9)
2012	Blood	16S rRNA	1652	Culture MAT	52.7%	97.2%	(102)
2012	Blood urine	16S rRNA	261	MAT IgM ELISA	94.4%	100%	(103)

These genes have also been used in MLST (multilocus sequence typing) for typing leptospira (104). Recently, amplification of the *rfb* gene which codes for LPS in leptospira has been found to be partially successful for determination of serovar by Silva *et al* (105). This is in agreement with the opinion stated by Patarakul K regarding the lack of similarity between leptospiral serovars and sequence information (106). The currently available PCRs detect leptospira DNA effectively as they are designed based on the conserved regions but are unable to further classify them into serovars. The advantages of PCR are that it is rapid and it can detect the organism even during the first week of illness before the appearance of antibodies and hence aids in early initiation of treatment. The limitations of PCR are that it is costly as it requires technical expertise and expensive infrastructure and is also labor – intensive. Further, it cannot identify the infecting serovar. This can be overcome by restriction endonuclease digestion of PCR end-products, direct sequencing of amplicons, and single strand confirmation analysis.

LAMP

LAMP (loop-mediated isothermal amplification) is an alternative method of DNA amplification with high specificity, efficiency and occurs under isothermal conditions. It employs a DNA polymerase and a set of four specially designed primers that recognise six DNA sequences on the target DNA (11). The final products of LAMP are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. These enable their simple, easy, selective detection by electrophoresis (12). The reaction results in the accumulation of 10^9 copies of target. The LAMP assay simply requires a laboratory water bath or heating block to maintain a constant temperature of 60–65°C,

making it particularly suitable for resource poor settings (6). Detection of products is done by agarose gel electrophoresis, real-time monitoring in an inexpensive turbidometer or in the form of a colour change when SYBR Green I, a fluorescent dsDNA intercalating dye is used which detects turbidity or a pellet. Moreover, the LAMP assay is not affected by polymerase inhibitors (12).

The advantages of LAMP assay are –

1. LAMP amplifies DNA with high efficiency under isothermal condition. It does not require a thermal cycler like PCR and can be done in a water-bath/ heating block at 65^0C.
2. It is a sensitive test with detection limit of a few copies (11).
3. The end products are a mixture of stem- loop DNAs which can be easily detected by naked eye by turbidity, fluorescent dye or colorimetry like antigen- antibody reactions (12).
4. LAMP is highly specific for target sequence due to recognition of target by six independent sequences in the initial stage and four independent sequences in the later stage.
5. LAMP is simple and easy to perform and less expensive as it requires only primers, a DNA polymerase and a water bath.
6. By combination with reverse transcription, LAMP can amplify RNA sequences with high efficiency.
7. LAMP is not affected by polymerase inhibitors and haem (11).

Lin *et al* in 2009 developed a LAMP assay targeting the LipL41 gene and reported the lower detection limit of 100 genome equivalents similar to PCR in mouse kidney sample. The assay was easier and inexpensive compared to PCR (13).

Sonthayanon *et al* developed another LAMP assay targeting the *rrs* gene and found the analytical sensitivity of 10 genome equivalents. They also performed clinical evaluation of the *rrs* LAMP and the LipL41 LAMP in blood samples from cases positive by culture and/or MAT, and in controls with other febrile illness. The sensitivity and specificity for *rrs* LAMP

was 43.6% and 83.5% respectively, while for the LipL41 LAMP the sensitivity and specificity was 37.6% and 90.2% respectively. This difference was not found to be statistically significant. Among patients who were positive for blood culture, the sensitivity for *rrs* and LipL41 LAMP assay were higher at 84.6% and 74.4% respectively. The poor sensitivities of both the LAMP assays found in the study were attributed to common use of over-the-counter antibiotics or presence of low number of organisms in blood below the detection limit of either assay. The authors suggested that additional clinical evaluation was necessary before LAMP could be used for diagnosis (6).

Since leptospiral DNA have been detected from urine both in early and late disease by PCR rather than in blood, Koizumi *et al* developed an *rrs* LAMP assay which can be used in heated urine samples. The lower detection limit of the assay was 2 and 10 genome equivalents per reaction under heat-denaturing and non-denaturing conditions. The performance of the assay was evaluated in urine of field mice. The assay was positive in 11 of 12 culture positive boiled urine and in 10 of 11culture positive urine pellet samples. The LAMP assay was also positive in two culture negative samples, suggesting a higher sensitivity than culture. As shedding of the leptospires occur intermittently, the authors suggested repeated testing using nucleic acid amplification (14).

In another study by Suwancharoen D *et al*, the detection limit was upto 10 to 100 copies using 16S rDNA as the target. In this study DNA from 22 pathogenic and 2 non-pathogenic leptospira and some other bacteria like *Enterococcus faecalis, Staphylococcus aureus, Proteus mirabilis* were used. The LAMP assay was compared to standard PCR and it was found that LAMP assay was 10-100 times more sensitive than PCR. The LAMP assay detected all the pathogenic leptospira but none of the non-pathogenic leptospira or other bacteria (15).

The summary of all these LAMP studies with their detection limit, sensitivity and specificity are given below in table 3.10.

Table 3.10: Summary of LAMP assay findings of the four research groups

Year	Sample	Target	Detection limit	Compared with	Comments	Reference
2009	Monkey kidney sample	LipL41 gene	100 genome equivalents	Real-time PCR	Detection limit same as PCR	(13)
2011	Blood	*rrs* gene	10 genome equivalents	Culture	Sensitivity 43.6% and Specificity 83.5%	(6)
		LipL41 gene			Sensitivity 37.6% and Specificity 90.2%	
2012	Urine of rats	*rrs* gene	2 to 10 genome equivalents	Culture, FlaB nested PCR	Specificity 66.7% and Sensitivity is 11 of 12 in boiled urine, 10 of 11 in urine pellets	(14)
2012	DNA of 22 pathogenic and 2 non-pathogenic leptospira	16S rDNA	10 to 100 copies	PCR	Sensitivity - 10-100 times more sensitive than PCR Specificity – 100% with pathogenic leptospira	(15)

Case definition of leptospirosis

The gold standard for diagnosis of leptospirosis being culture; it cannot be done on all samples. So a composite criteria is needed to make sure that no case of leptospirosis is missed. Such composite criteria are given by WHO, CDC, Faine's and modified Faine's criteria.

World Health Organisation – Definition (44)

According to World Health Organisation - The clinical features consist of an acute febrile illness with headache, myalgia (particularly calf muscle) and prostration associated with any of the following symptoms/signs:

Conjunctival suffusion
Anuria or oliguria
Jaundice
Cough, haemoptysis and breathlessness
Haemorrhage from the intestine and lung
Meningeal irritation
Cardiac arrhythmia or failure
Skin rash
Other common symptoms include nausea, vomiting, abdominal pain, diarrhoea, arthralgia

.

Laboratory criteria

Presumptive diagnosis:

A positive result of a rapid screening test such as IgM ELISA, latex agglutination test, lateral flow, dipstick etc.

Confirmatory diagnosis:

i. Isolation from blood or other clinical materials through culture of pathogenic leptospira

ii. A positive PCR result using a validated method (primarily for blood and serum in the early stages of infection).

iii. Fourfold or greater rise in titre or seroconversion in microscopic agglutination test (MAT) on paired samples obtained at least 2 weeks apart. A battery of *Leptospira* reference strains representative of local strains should be used as antigens in MAT.

Case classification

Suspected: A case that is compatible with the clinical description and a presumptive laboratory diagnosis.

Confirmed: A suspect case with a confirmatory laboratory diagnosis.

Centre for Disease Control and Prevention – Definition (107)

Clinical features are

i. History of fever within the last two weeks and

ii. At least two of the following features - myalgia, headache, jaundice, conjunctival suffusion and maculopapular rash.

iii. Or at least one of the following clinical findings - septic meningitis, gastro intestinal symptoms (abdominal pain, nausea, vomiting, diarrhoea), pulmonary symptoms (cough, breathlessness, haemoptysis), cardiac arrhythmias, renal insufficiency (anuria, oliguria), haemorrhage (intestinal, pulmonary, haematuria, hematemesis), jaundice with acute renal failure.

Laboratory Criteria for Diagnosis

Supportive: Any of the following

i. Microscopic agglutination test (MAT) has a titre of ≥ 200 but < 800 in one or more serum specimens.
ii. Demonstration of anti leptospira antibodies in a clinical specimen by indirect immunofluorescence.
iii. Demonstration of leptospira in a clinical specimen by darkfield microscopy
iv. Detection of IgM antibodies against leptospira in an acute phase serum specimen.

Confirmed: Any of the following

i. Isolation of leptospira in culture from a clinical specimen.
ii. Fourfold or greater rise in titre between acute and convalescent serum.
iii. Demonstration of leptospira in tissue by direct immunofluorescence.
iv. Microscopic agglutination test (MAT) shows a titre of ≥ 800 in one or more serum specimens.
v. Detection of pathogenic leptospira DNA (by PCR) from a clinical specimen.

Epidemiologic Linkage: This includes occupational, recreational or vocational exposure to animals or environments contaminated with animal urine like involvement in an exposure event (adventure race or flood).

Case Classification

Probable: A clinically compatible case with at least one of the following:

i. Involvement in an exposure event (adventure race, triathlon, flooding) with known associated cases.

ii. Presumptive laboratory findings, but without confirmatory laboratory evidence of leptospira infection.

Confirmed: A case with confirmatory laboratory results, as listed above.

These case definitions are not always practically feasible as culture and PCR both have low sensitivity and it is very difficult to get a paired serum sample from a patient with acute febrile illness like leptospirosis. Moreover, even if the paired serum is obtained, rise in titre is not demonstrable as the patient takes antimicrobial drugs in the course of illness.

Faine had developed a criteria for diagnosis of leptospirosis based on clinical, epidemiological and laboratory features (table 3.11). This criteria was modified by Shivakumar *et al* in Chennai to make it suitable for use in India (108) as given in table 3.12. The reason for modification is that rainfall should be taken into account as most outbreaks of leptospirosis occur during monsoon. Though microscopic agglutination test is the serological reference standard but it is complicated and difficult to perform. Hence, ELISA and slide agglutination test (SAT) which are simple and easy to perform have also been included in the criteria (109).

Table 3.11: Faine's criteria for diagnosis of leptospirosis (109)

Part A : Clinical data	Score
Headache	2
Fever	2
Temperature >39^0C	2
Conjunctival suffusion (bilateral)	4
Meningism	4
Muscle pain (especially calf muscle)	4
Conjunctival suffusion + meningism + muscle pain	10
Jaundice	1
Albuminuria or nitrogen retention	2
Part B: Epidemiological factors	
Contact with animals or contact with known contaminated water	10
Part C: Laboratory findings	
Isolation of leptospira in culture – diagnosis certain	
Positive serology MAT	
Leptospirosis endemic single positive low titre	2
Leptospirosis endemic single positive high titre	10
Leptospirosis non-endemic single positive low titre	5
Leptospirosis non-endemic single positive high titre	15
Rising titre in paired sera	25
Only one serological test should be scored	
Diagnosis of leptospirosis is Part A+ B ≥ 26 or Part A+ B+ C ≥ 25	

Table 3.12: Modified Faine's criteria for diagnosis of leptospirosis (109)

Part A : Clinical data	Score
Headache	2
Fever	2
Temperature >39^0C	2
Conjunctival suffusion (bilateral)	4
Meningism	4
Muscle pain (especially calf muscle)	4
Conjunctival suffusion + meningism + muscle pain	10
Jaundice	1
Albuminuria or nitrogen retention	2
Part B: Epidemiological factors	
Rainfall	5
Contact with contaminated environment	4
Animal contact	1
Part C: Laboratory findings	
Isolation of leptospira in culture – diagnosis certain	
IgM ELISA positive	15
SAT positive	15
MAT single high titre	15
MAT rising titre in paired sera	25
Only one serological test should be scored	
Diagnosis of leptospirosis is Part A+ B ≥ 26 or Part A+ B+ C ≥ 25	

Treatment

Leptospirosis is treated with supportive measures and antibiotics. The different antimicrobial agents that can be used for treatment of leptospirosis are penicillin, doxycycline, cefotaxime, ceftriaxone and azithromycin (110). Severe disease is treated with penicillin and ceftriaxone while mild disease is treated with doxycycline.

Table 3.13: Drugs used for the treatment of leptospirosis (24)

Severity of illness	Antibiotic	Dosage
Severe illness	Penicillin G	1.5MU IV 6 hourly
	Ceftriaxone	1gm IV once daily
	Ampicillin	0.5 – 1gm IV 6 hourly
Mild illness	Doxycycline	100mg twice daily
	Ampicillin	500 – 750mg 6 hourly

Other antibiotics which can be used for treatment of leptospirosis are azithromycin and amoxicillin. In a study by Phimda *et al*, the efficacy of azithromycin and doxycycline were similar for treatment of leptospirosis. However, azithromycin is more expensive than doxycycline, though the former has lesser adverse effects compared to doxycycline (111). Ghouse *et al* demonstrated that 72% of the patients having leptospira infection responded well to treatment with azithromycin and had complete cure (112). In some conditions, fluoroquinolones like ciprofloxacin has also been used for treatment of leptospirosis (113). The complications of leptospirosis like renal failure require other measures like haemodialysis (24).

Prevention

The prevention of leptospirosis involves the following –

1. Prevention of exposure: This includes wearing of shoes, gloves etc. while handling animals, swimming in safe water to prevent occupational and recreational exposure to leptospira (24).
2. Identification and controlling the source: Rain water should not be allowed to accumulate and prevention of contamination of water with urine of animals.
3. Rodent control: Rodent control is a definite approach for control of leptospirosis.
4. Control of disease in animals: The disease can be controlled in cattle by annual vaccination with killed vaccines.
5. Chemoprophylaxis: Doxycycline 200 mg in weekly dose is used for chemoprophylaxis in military personnel and people going to the jungle (30).

Materials and Methods

This study was approved by Institutional Review Board (IRB Min no 8109 dated 5.12.2012)

Patient recruitment

Adult patients, having fever of duration ≤ 15 days, whose sera were sent for leptopsirosis serology, were recruited based on the inclusion and exclusion criteria given below.

All the patients were recruited after obtaining an informed consent (Appendix I).

A clinical proforma was filled up as given in Appendix II at the time of sample collection.

Inclusion criteria:

Patients with the following were considered as suspected cases of leptospirosis

1. Individuals admitted with acute undifferentiated fever (≥100º F) of duration ≤ 15 days
2. Adult patients (≥ 18 years)
3. Malaria smear negative
4. Blood culture negative
5. Eschar negative

Exclusion criteria:

1. Individuals who had malaria, sepsis or enteric fever
2. Individuals who had fever with localizing signs
3. Duration of fever ≥ 16days
4. Individuals with fever and eschar
5. Outpatients

The study included two parts –

I. Validation of IgM ELISA for leptospirosis and scrub typhus

II. Evaluation of three assays for diagnosis of leptospirosis

Samples:

I. For Validation of IgM ELISA

A. Samples used to estimate the cut – off in normal population

Adults who were accepted as voluntary blood donors fulfilling the following criteria -

1. Adults ≥ 18years of age
2. Body weight ≥ 45 Kg
3. No acute illness in the past 30 days
4. Negative for HIV, Hepatitis B, Hepatitis C, malaria and syphilis
5. No history of tuberculosis in last 1 year

Four ml of blood from healthy blood donors was collected in EDTA tubes (BD Vacutainer, Franklin Lakes, NJ, USA).

This was used for obtaining plasma.

Four ml of blood was collected from 20 of the healthy donors in Serum tube with clot activator (BD Vacutainer, Franklin Lakes, NJ, USA).

This was used for obtaining serum.

B. For Validation of cut-off of ELISA determined

Serum samples were collected from proven cases of scrub typhus, sepsis, malaria, enteric fever and dengue. The details of these are given below in table 4.1

Table 4.1: Samples for validation of ELISA

Disease	Number of Sample
Scrub typhus patients having eschar *	50
Sepsis patients (blood culture positive)	20
Enteric fever patients (blood culture positive)	20
Malaria smear positive patients	17
Dengue serology positive patients	17

* Not used for validation of scrub typhus ELISA

These patients had no other illness except the one mentioned above. 4 ml of blood was collected from the patients in Serum tube with clot activator (BD Vacutainer, Franklin Lakes, NJ, USA).

These sera and plasma were tested for IgM and IgG antibodies to leptospira and IgM antibodies to scrub typhus by ELISA. The different tests performed are given in table 4.2

Table 4.2: ELISA tests performed

Test for leptospirosis	**Kit**
IgM ELISA	PanBio, (PanBio Ltd, Brisbane, Australia)
IgM ELISA	Virion serion (Serion Immundiagnostics, Wurzburg, Germany)
IgG ELISA	Virion serion (Serion Immundiagnostics, Wurzburg, Germany)
IgM ELISA	InBios (InBios International Inc, Seattle, WA)
Test for scrub typhus	**Kit**
IgM ELISA	InBios (InBios International Inc, Seattle, WA)

II. Evaluation of assays for diagnosis of leptospirosis

Four ml of blood was collected from the patients with suspicion of leptospirosis in Serum tube with clot activator (BD Vacutainer, Franklin Lakes, NJ, USA). The following tests (table 4.3) were performed with the serum samples of the patients with acute febrile illness:

Table 4.3: Tests for leptospirosis

Test for leptospirosis	Timing of sample
IgM ELISA (PanBio)	Paired sera: Acute & convalescent (≥ 7 days after obtaining the acute sample)
Nested PCR	Duration of illness ≤ 15 days
LAMP assay	Duration of illness ≤ 15 days

Sample size:

This was a pilot study as not much data is available on the prevalence of leptospirosis in India in general and this area in particular. Total 150 individuals who were suspected to be suffering from leptospira infection were enrolled in this study as per the inclusion and exclusion criteria elucidated above.

Study period:

December 2012 to July 2014

Separation of serum and plasma

Serum was separated by centrifugation at 2500 rpm for 10 minutes at 4^0C. The serum was stored at -70^0C in two aliquots. Plasma was separated by centrifugation at 2500 rpm for 10 minutes at 4^0C.

Serology

All ELISA were performed according to the manufacturer's instructions. Briefly the procedure of each is described below -

Leptospira IgM ELISA by PanBio (PanBio Ltd, Brisbane, Australia)

Procedure

1. The required number of microwells were taken out and placed into the strip holder.
2. Six microwells were labeled and kept for negative control, reactive control, calibrator in triplicate and in – house control.
3. Other wells were marked for the samples.
4. Negative control, reactive control, calibrator in triplicate, in – house control and patient samples were diluted. 90µl of sample diluent was added to 10µl of serum and mixed well. From this 20µl of the diluted serum was added to 180µl sample diluent and mixed well to get a final dilution of 1: 100.
5. Then, 100µl of diluted patient samples, controls and calibrator were put into their respective wells.
6. The plate was incubated at 37ºC for 30 minutes.
7. After incubation, the wells were washed well six times with diluted wash buffer taking 350µl each time for each well.
8. Then, 100µl of HRP (horse radish peroxidase) conjugated anti-human IgM was added to each well.
9. The plate was again incubated at 37ºC for 30 minutes.
10. After incubation the wells were washed well six times with diluted wash buffer taking 350µl each time for each well.
11. After that, 100µl of TMB (tetramethylbenzidine) was put into each well.

12. The plate was incubated at 20-25^{0}C for 10 minutes.

13. Finally, 100µl of stop solution was added into each well and mixed well.

Reading

Within 30 minutes, the absorbance of each well was read at a wavelength of 450 nm with a reference filter of 600-650 nm.

Calculation

1. The average absorbance of the triplicates of the calibrator was calculated.
2. Cut-off Value = Average of Calibrator x Calibration factor
3. Index value = Sample Absorbance/Cut-off Value
4. Result in PanBio Units was calculated by multiplying the index value by 10.

Interpretation

The negative control, positive control and calibrator control readings were taken and calculations were done.

For each sample the result was calculated in PanBio units.

The results for all samples in PanBio units were entered in the excel sheet.

Leptospira IgM ELISA (Virion serion, Serion Immundiagnostics, Wurzburg, Germany)

Procedure

1. The microwells were taken out and placed in the holder and labeled.
2. The wells were marked as one for blank, one for negative control and two for standard serum in duplicate and the rest for samples.
3. The samples were diluted in sample dilution buffer with rheumatoid factor-absorbent. The rheumatoid factor-absorbent was first diluted in dilution buffer as 1:4 to which samples were added and diluted as 1:100.
4. Then, 100µl of diluted patient samples, controls and standard serum were put into their respective wells.
5. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
6. After incubation, the plate was washed well with wash buffer taking 350µl each time for each well for six times.
7. Then, 100µl of IgM–conjugate was added to all wells except blank well.
8. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
9. After incubation, the plate was washed well with wash buffer taking 350µl each time for each well for six times.
10. After that, 100µl substrate solution was added to each well.
11. The plate was again incubated at 37ºC for 60 minutes in a moist chamber.
12. Finally, 100µl of stop solution was added into all wells and mixed well for stopping the reaction.

Reading

The OD (optical density) reading was taken within 60 minutes at 405 nm. The reference wave length was at 650 nm.

Calculation

The test was valid if -

i. The substrate blank had an OD < 0.25.

ii. The negative control was negative.

iii. The mean OD value of the standard serum after subtraction of blank was within the validity range, which was given on the lot specific quality control certificate of the kit.

iv. The variation of OD values of the standard serum was not higher than 20%.

Interpretation

The negative control and standard serum reading were taken and calculations were done.

If the test was valid the OD values of the samples were entered in the excel sheet.

Leptospira IgG ELISA (Virion serion, Serion Immundiagnostics, Wurzburg, Germany)

Procedure

1. The microwells were taken out and placed in the holder and labeled.
2. The wells were labeled as one for blank, one for negative control and two for standard serum in duplicate and the rest for samples.
3. The samples were diluted in sample dilution buffer to get a final dilution of 1:100.
4. Then, 100µl of diluted patient samples, controls and standard serum were put into their respective wells.
5. The plate was then incubated at 37ºC for 60 minutes in a moist chamber.
6. The plate was washed well with wash buffer taking 350µl each time for each well for six times.
7. After that, 100µl of IgM–conjugate was added to all wells except blank.
8. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
9. It was washed well with wash buffer taking 350µl each time for each well for six times.
10. Then, 100µl substrate solution was added to each well.
11. The plate was incubated at 37ºC for 60 minutes in moist chamber.
12. Finally, 100µl of stop solution was added into all wells and mixed well for stopping the reaction.

Reading

The OD reading was taken within 60 minutes at 405 nm against substrate blank, reference wave length was at 650 nm.

Calculation

The test was valid if -

i. The substrate blank had an OD < 0.25.

ii. The negative control was negative.

iii. The mean OD value of the standard serum after subtraction of blank was within the validity range, which was given on the lot specific quality control certificate of the kit.

iv. The variation of OD values of the standard serum was not higher than 20%.

Interpretation

The negative control and standard serum reading were taken and calculations were done.

If the test was valid the OD values of the samples were entered in the excel sheet.

Leptospira IgM ELISA (InBios, InBios International Inc, Seattle, WA)

Procedure

1. The microtitre wells were taken out and labeled.
2. Positive and negative controls were assayed in duplicate. So, two wells were taken for positive control, two wells for negative control and other wells were marked for samples.
3. The test sera were diluted to 1:100 by using the sample dilution buffer and mixed thoroughly.
4. Then, 100µl of the 1/100 diluted test sera and controls were put in each well.
5. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
6. It was washed well with wash buffer taking 350µl each time for each well for six times.
7. After that, 100µl of HRP conjugate was added to each well.
8. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
9. It was washed well with wash buffer taking 350µl each time for each well for six times.
10. Then, 150µl of EnWash solution was added per well into all wells.
11. The plate was incubated at 20-25^{0}C for 5 minutes.
12. It was washed well with wash buffer taking 350µl each time for each well for six times.
13. Then, 100µl of liquid TMB substrate was added into all wells.
14. The plate was again incubated at 20-25°C for 10 minutes.
15. In the end, 50µl of stop solution was added into all wells and incubated at room temperature for 1 minute.

Reading

The readings were taken at the wave length of 450nm.

Calculation

The test was valid if

i. OD of Negative control < 0.1

ii. OD of Positive control > 0.5

iii. Discrimination capacity which is expressed as the ratio of

OD of positive control (PC)/ OD of negative control (NC) ≥ 5.

Interpretation

The negative control and positive control readings were taken and calculations were done.

If the test was valid the OD values of the samples were entered in the excel sheet.

Scrub typhus IgM ELISA (Inbios, Inbios International Inc, Seattle, WA)

Procedure

1. The microtitre wells were taken out, put in the holder and labeled.
2. Positive and negative controls were assayed in duplicate. So two wells for positive control, two wells for negative control and other wells were marked for samples.
3. The test sera were diluted to 1:100 by using the sample dilution buffer and mixed thoroughly.
4. Then, 100µl of the 1/100 diluted test sera and controls were put in each well.
5. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
6. It was washed well with wash buffer taking 350µl each time for each well for six times.
7. After that, 100µl of HRP conjugate was added to each well.
8. The plate was incubated at 37ºC for 60 minutes in a moist chamber.
9. It was washed well with wash buffer taking 350µl each time for each well for six times.
10. Then, 150µl of EnWash solution was added per well into all wells.
11. The plate was incubated at 20-25^{0}C for 5 minutes.
12. It was washed well with wash buffer taking 350µl each time for each well for six times.
13. To each well 100µl of liquid TMB substrate was added.
14. Then, the plate was incubated at 20-25°C for 10 minutes.
15. Finally, 50µl of stop solution was added into all wells and incubated at room temperature for 1 minute.

Reading

The readings were taken at the wave length of 450nm.

Calculation

The test was valid if

i. OD of Negative control < 0.1

ii. OD of Positive control > 0.5

iii. Discrimination capacity which was expressed as the ratio of

OD of positive control (PC)/ OD of negative control (NC) ≥ 5

Interpretation

The negative control and positive control readings were taken and calculations were done.

If the test was valid the OD values of the samples were entered in the excel sheet.

Polymerase Chain Reaction

Serum samples of the patients were used for PCR.

DNA extraction from serum samples:

DNA was extracted using the QIAamp blood mini kit (Qiagen, Hilden, Germany) from the serum samples as per the manufacturer's instructions for blood and body fluid protocol with the help of spin column. The method used was as follows -

1. Qiagen Proteinase K (20µl) was taken in the bottom of a 1.5 ml microcentrifuge tube.
2. To it, 200µl serum sample was added.
3. Then, 200µl Buffer AL (lysis buffer) was added to the sample in the same tube.
4. These substances were mixed well by pulse vortexing for 15 seconds.
5. The tube was incubated at 56^0C for 10 minutes.
6. After this, the tube was briefly centrifuged to remove the drops from inside the lid.
7. To the tube, 200µl of absolute alcohol was added.
8. Again, it was mixed well by pulse vortexing for 15 seconds.
9. Then, the tube was briefly centrifuged to remove the drops from inside the lid.
10. The mixture was placed in the QIAamp spin column with a 2 ml collection tube without wetting the rim and the cap was closed.
11. The spin column was centrifuged at 8000 rpm for 1 minute.
12. The QIAamp spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.
13. The QIAamp spin column was carefully opened and 500µl Buffer AW1 (Wash buffer) was added to it without wetting the rim and the cap was closed.
14. It was centrifuged at 8000 rpm for 1 minute.

15. The QIAamp spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.
16. The QIAamp spin column was carefully opened and 500µl Buffer AW2 (Wash buffer) was added without wetting the rim and the cap was closed.
17. It was centrifuged at full speed of 14,000 rpm for 3 minutes.
18. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded.
19. The QIAamp spin column was carefully opened and 200µl Buffer AE (eluting buffer) was added to it.
20. The tube was incubated at room temperature for 1 minute.
21. It was then centrifuged at 8000 rpm for 1 minute and the spin column was discarded.
22. The 1.5 ml centrifuge tube contained DNA which was labeled and stored at -70^0C in two aliquots – one aliquot was used for PCR and the other one was used for LAMP assay.

Polymerase Chain Reaction for leptospirosis

The DNA was stored at -70^{0}C.

Nested PCR

Gene*: rrs* gene

Amplicon size: 547bp

The primer sequence used for nested PCR to detect a 547bp fragment of the *rrs* gene of pathogenic leptospira was as described by Boonsilp *et al* (9). The primer sequence was as given below in table 4.4

Table 4.4: Primer used for Leptospira PCR

Primer	Sequence
rrs-outer-F:	5'-CTCAGAACTAACGCTGGCGGCGCG-3'
rrs-outer-R	5'-GGTTCGTTACTGAGGGTTAAAACCCCC-3'
rrs-inner-F	5'-CTGGCGGCGCGTCTTA-3'
rrs-inner-R	5'-GTTTTCACACCTGACTTACA-3'

The thermal cycler 'Veriti' 96 well Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used to optimize the primer concentrations required.

Reaction Volume for PCR: 50µl in each run

Standardisation of PCR:

The PCR was standardised using the control strains of *Leptospira interrogans* obtained from Regional Medical Research Centre, Port Blair, India. The strains obtained were as follows (table 4.5)

Table 4.5: Strains of leptospira used for standardisation of PCR

Genomospecies	Serogroup	Serovar	Strain
Leptospira interrogans	Pomona	Pomona	Pomona
Leptospira interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Leptospira interrogans	Sejroe	Hardjo	Hardjo

Standardisation of PCR was done by varying magnesium chloride concentrations from 2mM to 5mM with increasing 0.5 mM / reaction. The primer concentration was also varied with the different concentration of 5pmol, 10pmol and 20pmol of each primer.

Subsequently, the optimal conditions were determined.

It was a nested PCR.

In the first cycle, the reaction mixture contained 2X PCR mix (Thermo Fisher Scientific, Marietta, USA), 20 pmol of each of the primers, 4mM Magnesium chloride and water along with 5µl of DNA.

In the second cycle, the reaction mixture contained 2X PCR mix (Thermo Fisher Scientific, Marietta, USA), 20 pmol of each of the primers, 4mM Magnesium chloride and water along with 5µl of the amplified product from the first cycle.

Table 4.6: Cycling parameters

Run	Temperature	Time	Cycle
First Run	95°C	2 minutes	1 cycle
	95°C	10 seconds	40 cycles
	67°C	15 seconds	
	72°C	30 seconds	
	72°C	7 minutes	1 cycle
Second Run	95°C	2 minutes	1 cycle
	95°C	10 seconds	40 cycles
	55°C	15 seconds	
	72°C	30 seconds	
	72°C	7 minutes	1 cycle

Detection of the amplicon: The *rrs* gene amplified product (10µl) was electrophoresed in a submarine electrophoresis unit using a 2% agarose gel containing ethidium bromide (10µg/ml). The PCR product was visualized using a gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

The positive and negative controls of each run were checked and the run was validated. Then the results of the samples were taken and entered in the excel sheet.

Genomic Sequencing

Randomly, two amplified products for *rrs* gene were subjected to sequencing reaction to confirm the appropriateness of the target amplified. The sequencing procedure includes the following steps -

Pre – Clean up

It was done according to the protocol given by HighPrep PCR (Magbio Genomics, Inc. Gaithersburg, Canada)

1. The HighPrep PCR reagent was brought to room temperature for at least 30 min before use. It was shaken thoroughly to fully resuspend the magnetic beads.
2. To 20µl of PCR amplicon, 36µl of HighPrep PCR reagent was added and mixed thoroughly with a pipette 6-8 times in a 1.5 ml eppendorf tube.
3. It was incubated at room temperature for 5 minutes.
4. The sample tubes were placed on a magnetic separation device for 3 minutes until the solution clears.
5. With the samples still on the magnetic separation device, the supernatant was removed and discarded with a pipette.
6. Then, 200µl of 70% ethanol was added to each sample tube without removing the tubes from the magnetic separation device.
7. The tube was incubated on the magnetic separation device for 30 seconds at room temperature.
8. The clear supernatant was removed and discarded with a pipette.
9. Again, 200µl of 70% ethanol was added to each sample tube without removing the tubes from the magnetic separation device.
10. It was incubated on the magnetic separation device for 30 seconds at room temperature.

11. The clear supernatant was removed and discarded with a pipette.
12. The beads were dried by incubating at room temperature for 5 minutes with the plate still on the magnetic separation device.
13. The samples were removed from the magnetic separation device.
14. Finally, 40 µl of water was added to each tube and it was mixed thoroughly five times with a pipette.
15. The samples were placed back on the magnetic separation device and incubated at room temperature for 1 minute.
16. The elute was taken in new tubes and used for PCR for sequencing and gel documentation to detect the product.

PCR for Sequencing

Reaction volume: 10µl

The reaction mixture contained RR mix, buffer, 1 pmol concentration of one primer (either forward or reverse), 2µl of the product DNA after pre-clean up, and water.

Table 4.7: Cycling parameters

Temperature	Time	Cycle
96°C	15 seconds	25 cycles
50°C	20 seconds	
60°C	4 minutes	

Post Clean up

1. DTR reagent (10µl) was taken in a 1.5ml eppendorf tube.
2. To it, 10µl of amplified PCR product and 40µl of 85% ethanol were added and mixed well ten times with a pipette.
3. The tube was incubated for 5 minutes at room temperature on the magnetic separation device.
4. The supernatant was discarded with a pipette.
5. Then, 100µl of 85% ethanol was added to each tube keeping it on the magnetic separation device without mixing.
6. It was incubated for 2 minutes at room temperature on the magnetic separation device and the supernatant was discarded.
7. Again, 100µl of 85% ethanol was added to each tube keeping it on the magnetic separation device without mixing.
8. It was incubated for 2 minutes at room temperature on the magnetic separation device and the supernatant was discarded.
9. The cap was opened and the beads were allowed to dry by incubating at room temperature for 10 minutes.
10. Finally, 40µl of injection solution was added to it and mixed well.
11. The tubes were incubated at room temperature for 5 minutes on the magnetic separation device.
12. The clear supernatant was taken and loaded in the sequencing plate.

Sequencing

The sequencing plate was loaded in the sequencer to obtain the genomic sequences. The ABI 310 Genetic analyser (Applied Biosystems, Foster City, CA, USA) was used to enumerate the sequences.

Analysis

The sequence obtained was visualised and edited using Finch TV (Perkin Elmer, Seattle, WA) to attain the final sequence for analysis.

The homology of the sequence obtained with that of the existing leptospira sequence in the Gene Bank was performed using the basic local alignment search tool (BLAST, available from www.ncbi.nlm.nih.gov/BLAST) programme with the available standard reference sequences in the Gene Bank for homology.

The Megablast format of this programme which searches for highly similar sequences was used for analysing the sequences.

LAMP (loop – mediated isothermal amplification) assay for leptospira

The LAMP assay was performed using the protocol and primer sequence provided by Dr WM Ching, Senior Scientist, Naval Medical Research Centre, Silver Spring, MD, USA. As the primer sequence has not yet been published, the primer sequence is not revealed. However, it has been validated by Dr. Ching's research group.

Target: LipL32, LipL41 gene

Primer Mix preparation - 3µl

Table 4.8: Primer concentration used in LAMP assay

Primer	Stock Concentration	Volume added (µl)
L32-F3	25	0.2
L32-B3	25	0.2
L32-FIP	100	0.4
L32-BIP	100	0.4
L32-LF	100	0.2
L32-LB	100	0.2
L41-F3	25	0.2
L41-B3	25	0.2
L41-FIP	100	0.4
L41-BIP	100	0.4
L41-LB	100	0.2

Reaction volume - 25µl

Master mix preparation: for 5 ml

Table 4.9: Master mix for LAMP

Component	Volume
10X Thermopol (NEB)	1 ml
Betaine (5M)	1.6 ml
Magnesium sulphate (1M)	60µl
Each dNTP (100mM)	140µl
Water	1.78 ml

Components of the LAMP Reagent: per 25µL reaction

Table 4.10: Components of LAMP assay

Component	Volume
2x Master mix	12.5µl
Primer mix	3µl
Water	3.5µl
*Bst*polymerase (from NEB)	1µl (8 units)
DNA Sample	5µl

In each run a positive control which was *Leptospira interrogans* strain Icterohaemorrhagiae obtained from Regional Medical Research Centre, Port Blair, India and a negative control were used.

Cycling condition: 63^{o}C for 60 minutes.

Detection of the LAMP products:

1. Visual detection for turbidity –

   The positive sample should be turbid while the negative sample should be clear.

2. Centrifugation – The tube was centrifuged at 14,000rpm for 1 minute for pellet formation.

   The positive sample had a tear – drop pellet while in the negative sample no pellet was seen.

3. Gel documentation: It was done by gel electrophoresis using a 2% agarose gel containing ethidium bromide (10µg/ml). The product was visualized using a gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

   The positive reaction had bands while the negative reaction had no band. The multiple banding pattern in LAMP assay was compared with that of the control strain.

The readings for the positive and negative controls were taken and the run was validated. Then the results for the samples were taken and entered in the excel sheet.

Data analysis

The OD values of the ELISA were all entered in the excel spreadsheet (Microsoft Office, Redmond, Washington, USA). The geometric mean (GM) and the standard deviation (SD) were calculated using excel spreadsheet. The cut-off was calculated as GM+3SD.

All samples which were positive by PCR or LAMP twice were considered positive by the respective tests.

Case definition: The case definition used in this study included the samples which were positive by PCR or LAMP or fulfilling modified Faine's (109) criteria for leptospirosis were taken as cases of leptospirosis.

All data was entered in the clinical proforma and then in the master-table in excel spreadsheet.

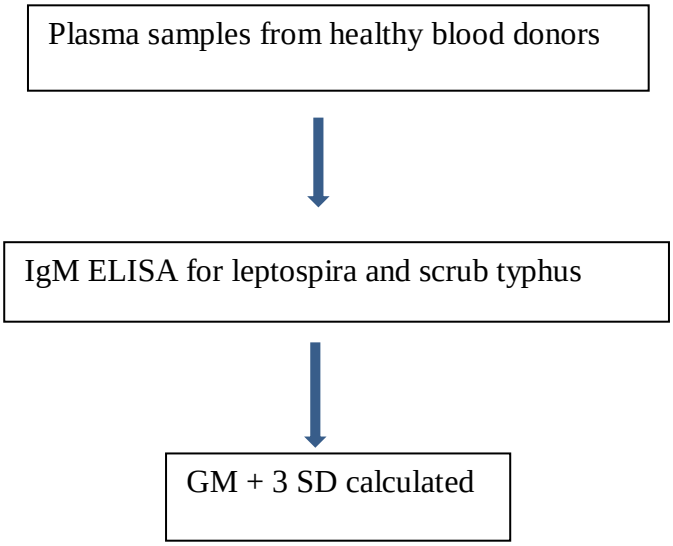
Statistical analysis

The statistical analysis of the clinical data was done using STATA version 13. The data were summarised using mean along with standard deviation for continuous variables, and frequency along with percentages for categorical variable. Chi square test was used to check the categorical variables association and p value <0.05 was taken as significant.

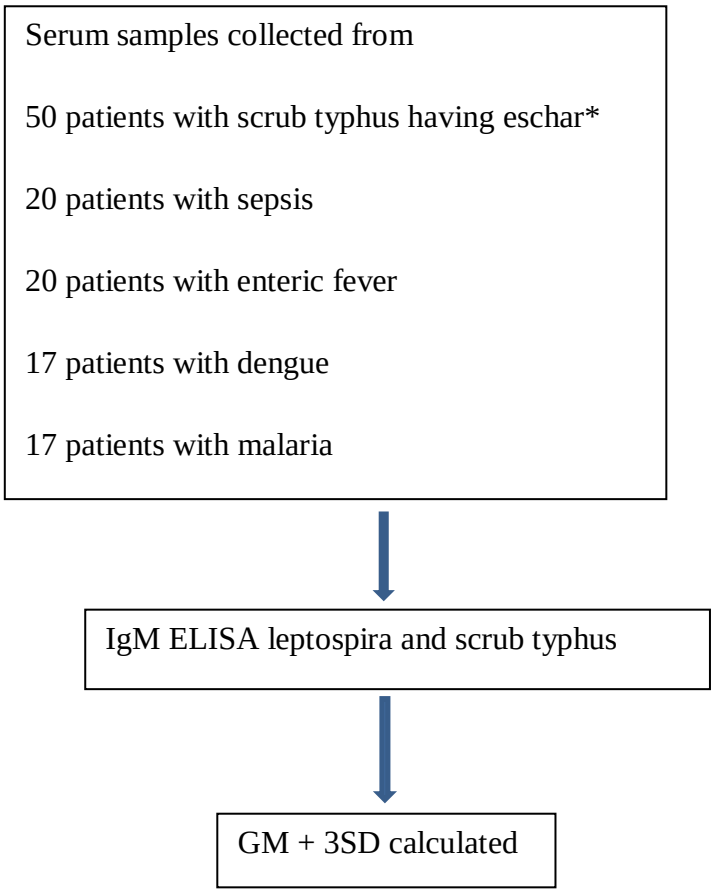
The diagnostic accuracies (sensitivity and specificity) of ELISA, PCR, LAMP were evaluated using Latent Class Analysis (LCA). The LCA was done to evaluate the true status of disease using the available three tests. The derived result from this technique was considered as gold standard and diagnostic accuracies were given.

I. For Validation of cut –off for ELISA

A. Determination of cut – off in healthy individuals

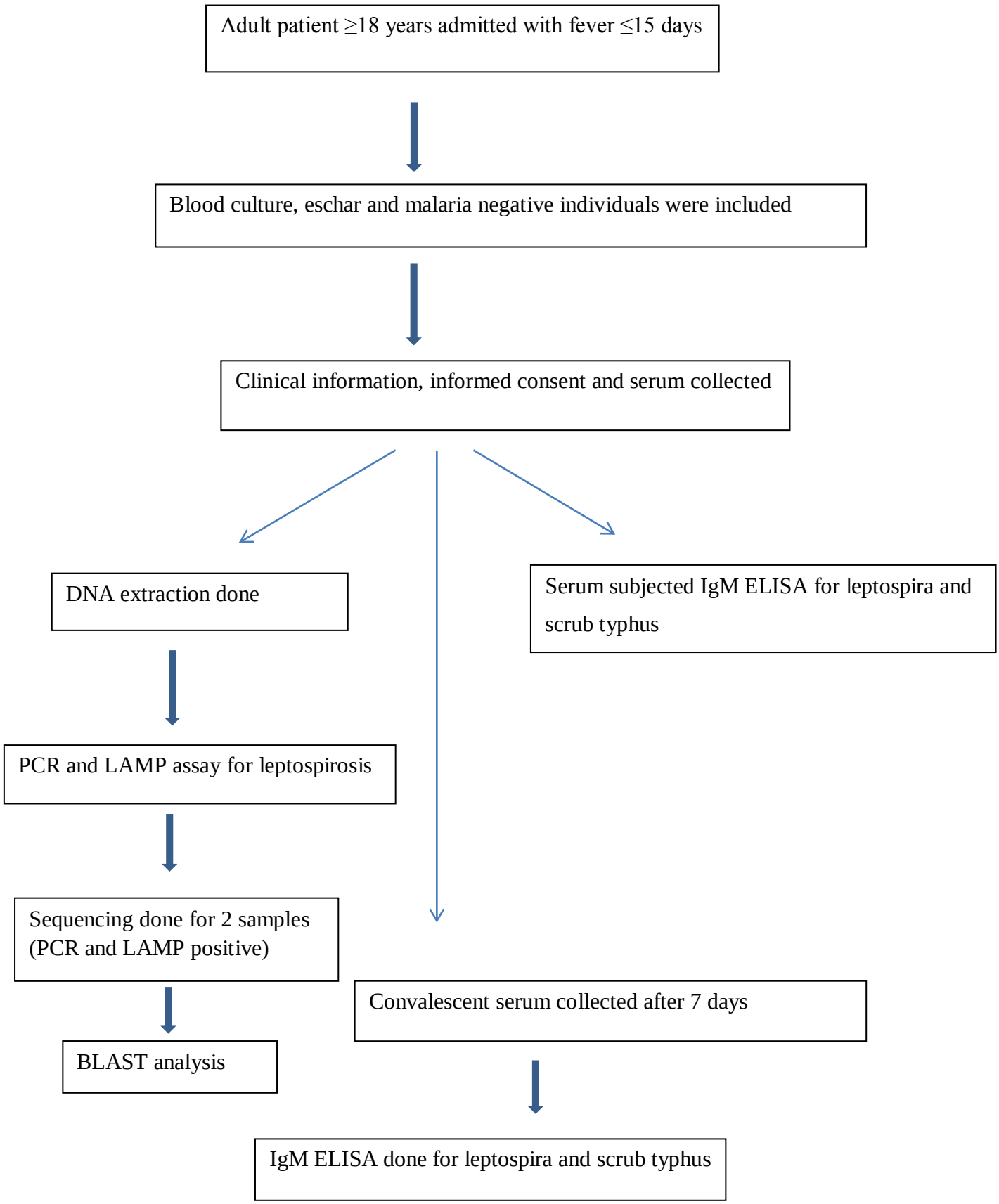


B. Determination of diagnostic cut - off



* Was not used for validation of scrub typhus ELISA

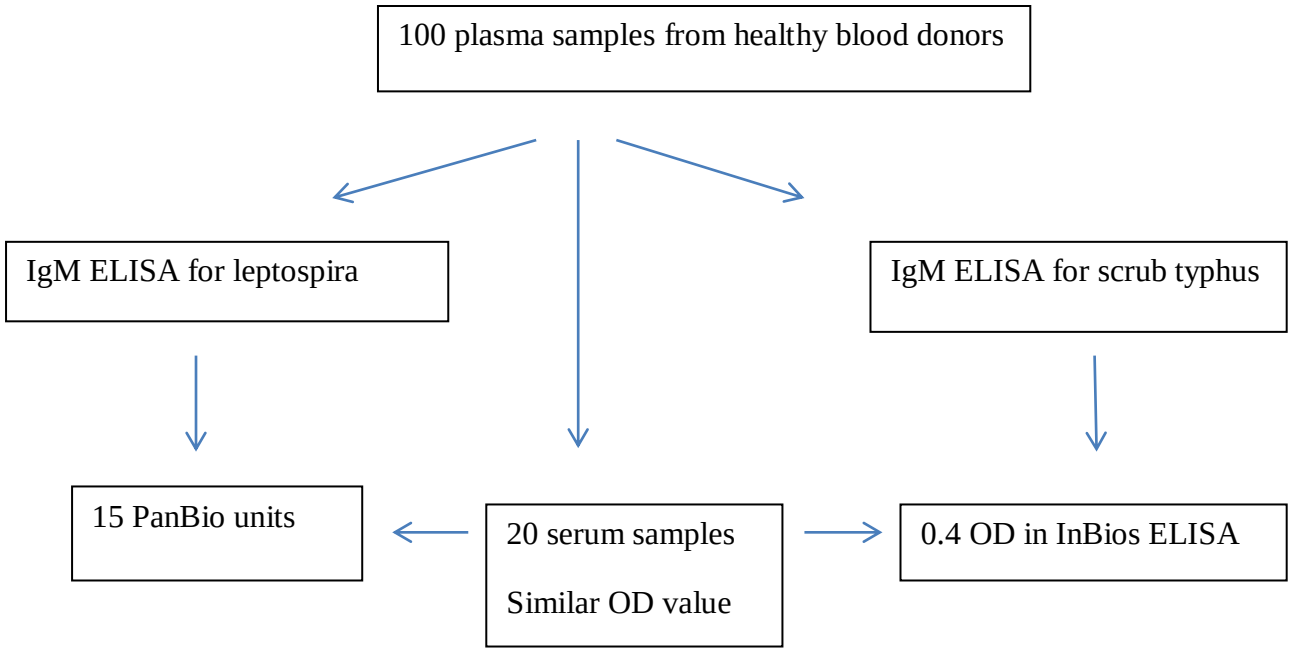
II. Evaluation of tests for diagnosis of leptospirosis



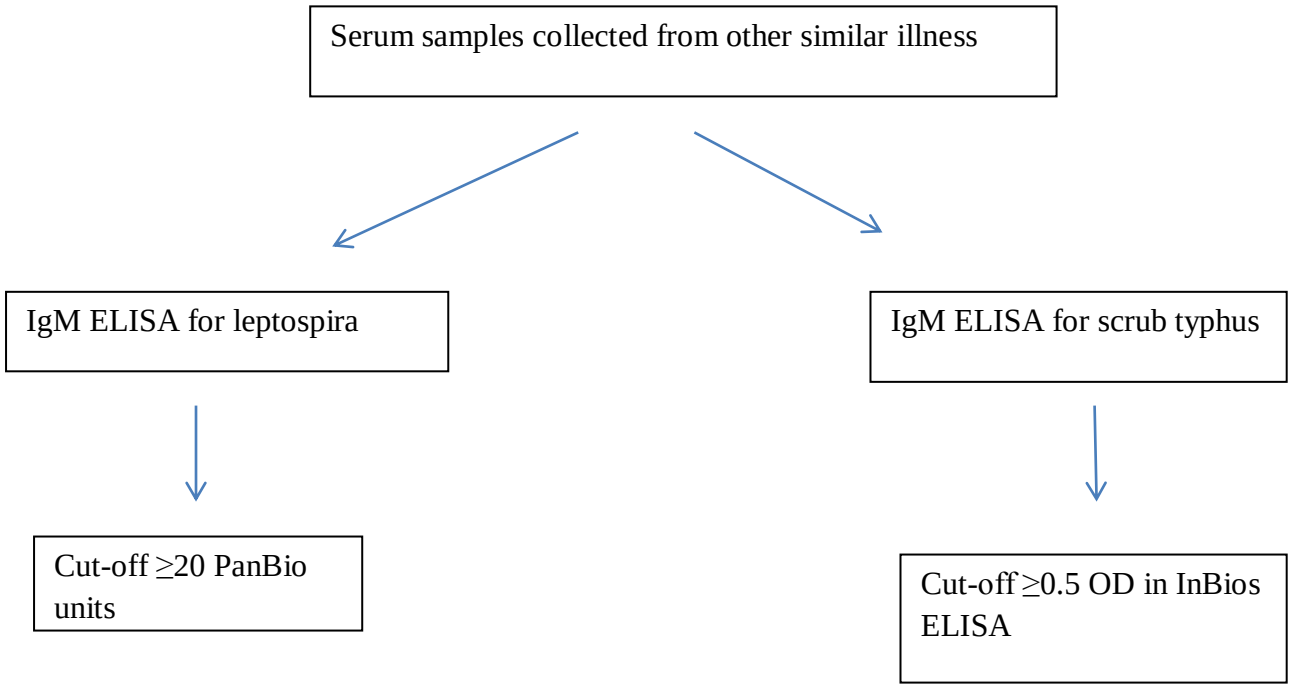
Results

I. For Validation of cut –off for ELISA

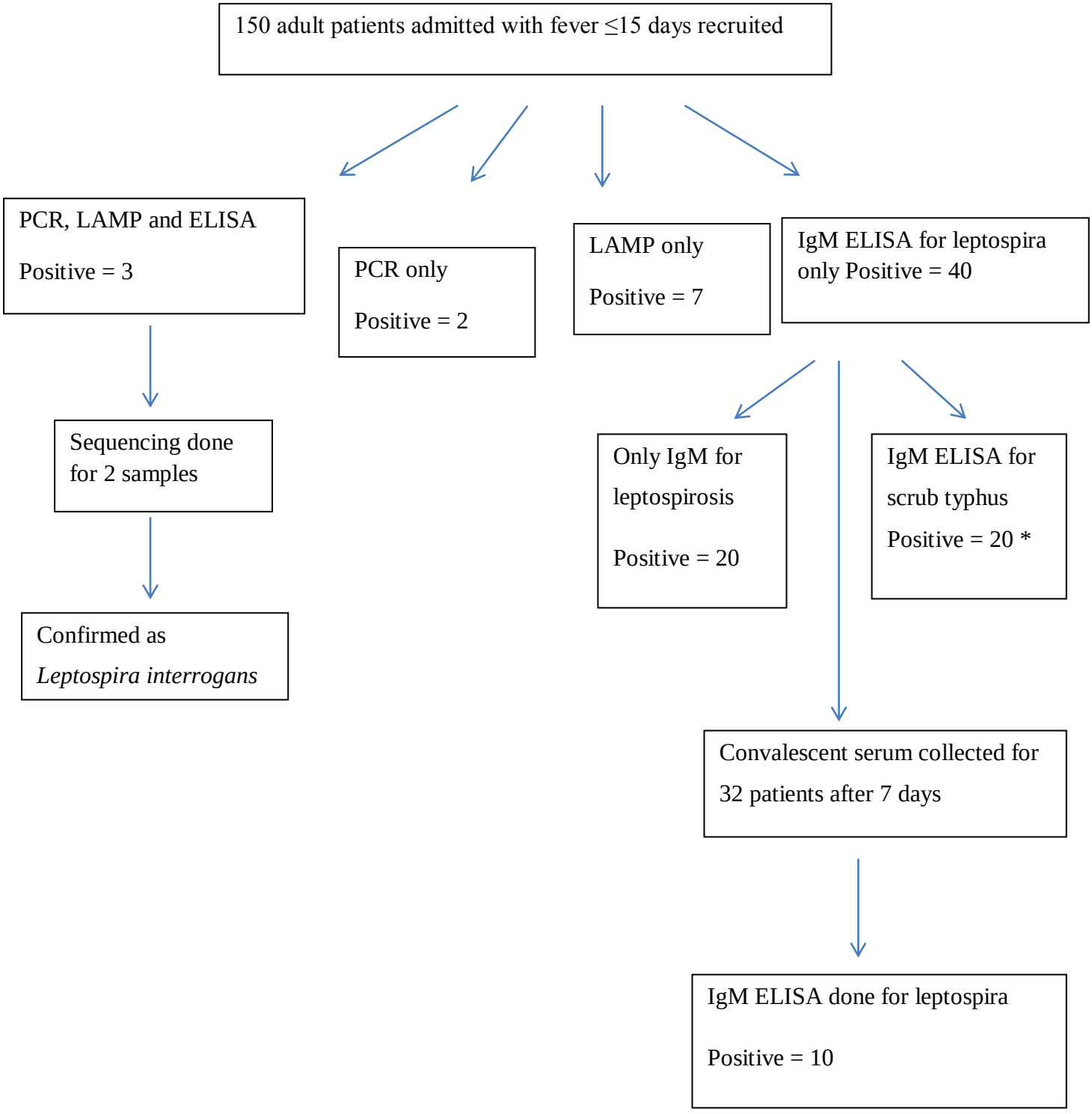
A. Determination of cut – off in healthy individuals



B. Determination of diagnostic cut - off



II. Evaluation of tests for diagnosis of leptospirosis



* Two more samples were positive for IgM ELISA for scrub typhus. One sample was positive by both PCR and LAMP assay and the other was positive only by the LAMP assay.

The plasma samples from healthy blood donors were used for determination of cut-off value of optical density (OD) for the different ELISAs and the same is as given below (table 5.1)

Table 5.1: ELISA cut – off value – Healthy blood donors

	Leptospira PanBio IgM (PanBio units)	Leptospira Virion serion IgM (OD)	Leptospira Virion serion IgG (OD)	Leptospira InBios IgM (OD)	Scrub typhus InBios IgM (OD)
Geometric Mean (GM)	3.974	0.198	0.483	0.017	0.072
Standard deviation (SD)	3.5	0.157	0.367	0.035	0.082
2 SD	7	0.314	0.734	0.07	0.164
3 SD	10.5	0.471	1.101	0.105	0.246
GM + 2 SD	10.974	0.512	1.217	0.087	0.236
GM + 3 SD	14.474	0.669	1.584	0.122	0.318
Cut-off	15	0.7	1.6	0.2	0.4

Serum samples from 20 healthy donors had similar OD values as the plasma.

The ELISA was validated using serum samples from patients having scrub typhus with eschar (n=50), sepsis (n=20), enteric fever confirmed by blood culture (n=20), malaria (n=17) and dengue (n=17). Table 5.2 shows the final cut-off determined after proper validation of the ELISA. Samples from patients with scrub typhus with eschar were not used for validation of scrub typhus IgM ELISA.

Table 5.2: Diagnostic cut-off for ELISA

	Leptospira PanBio IgM (PanBio units)	Leptospira Virion serion IgM (OD)	Leptospira Virion serion IgG (OD)	Leptospira InBios IgM (OD)	Scrub typhus InBios IgM (OD)
Geometric Mean (GM)	3.771	0.145	0.699	0.063	0.09
Standard deviation (SD)	5.141	0.129	0.666	0.313	0.127
2 SD	10.282	0.258	1.332	0.626	0.254
3 SD	15.423	0.387	1.998	0.939	0.381
GM + 2 SD	14.053	0.403	2.031	0.689	0.344
GM + 3 SD	19.194	0.532	2.697	1.002	0.471
Final Cut-off	20	0.7	2.7	1	0.5

For evaluation of the tests for diagnosis of leptospirosis, 150 patients with fever ≤15 days were recruited. Among 150 patients, 97 (64.7%) were male and 53 (35.3%) were female (Figure 5.1)

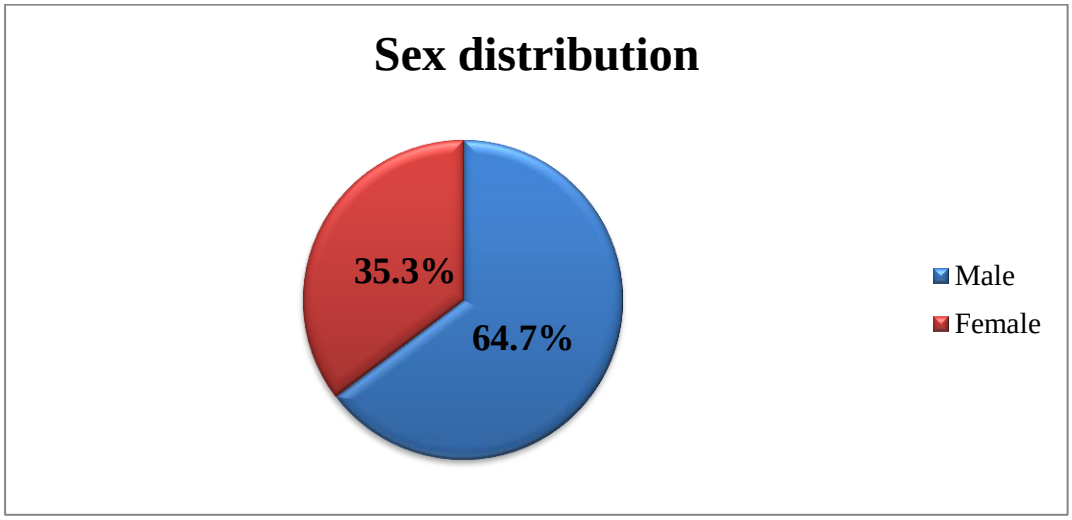


Figure 5.1: Sex distribution of the patients recruited

Among the patients recruited 42(28%) were between 18-30 years, 40(26.7%) were between 31-45 years, 45(30%) were between 46-60 years and 25(16.7%) were above 60 years of age (Figure 5.2)

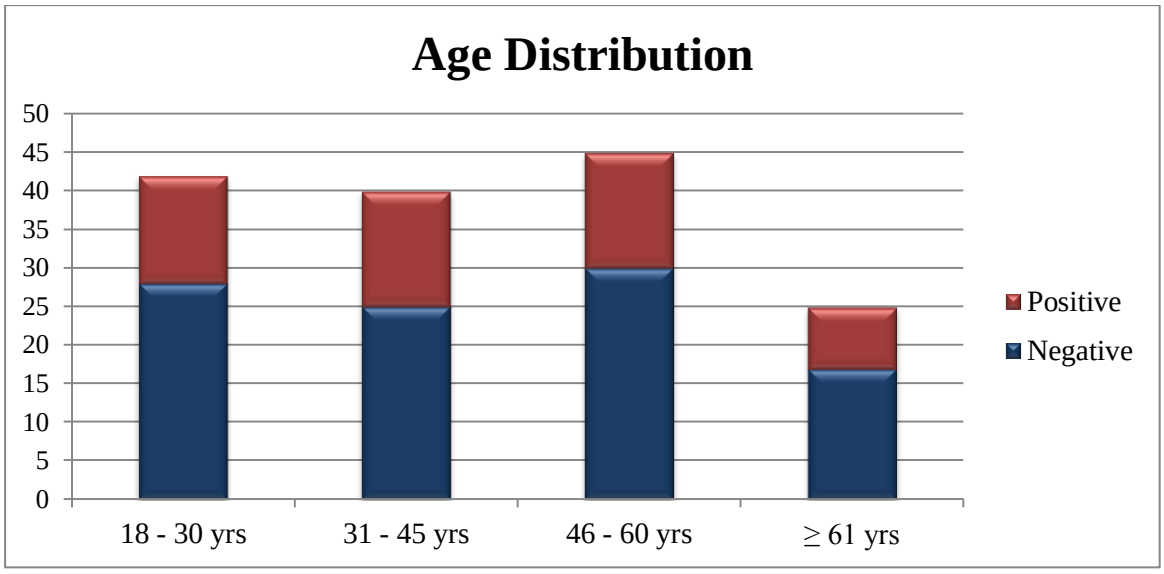


Figure 5.2: Age distribution of the study patients

Majority of the patients recruited were from Tamil Nadu (60%) and Andhra Pradesh (31.3%) (Figure 5.3)

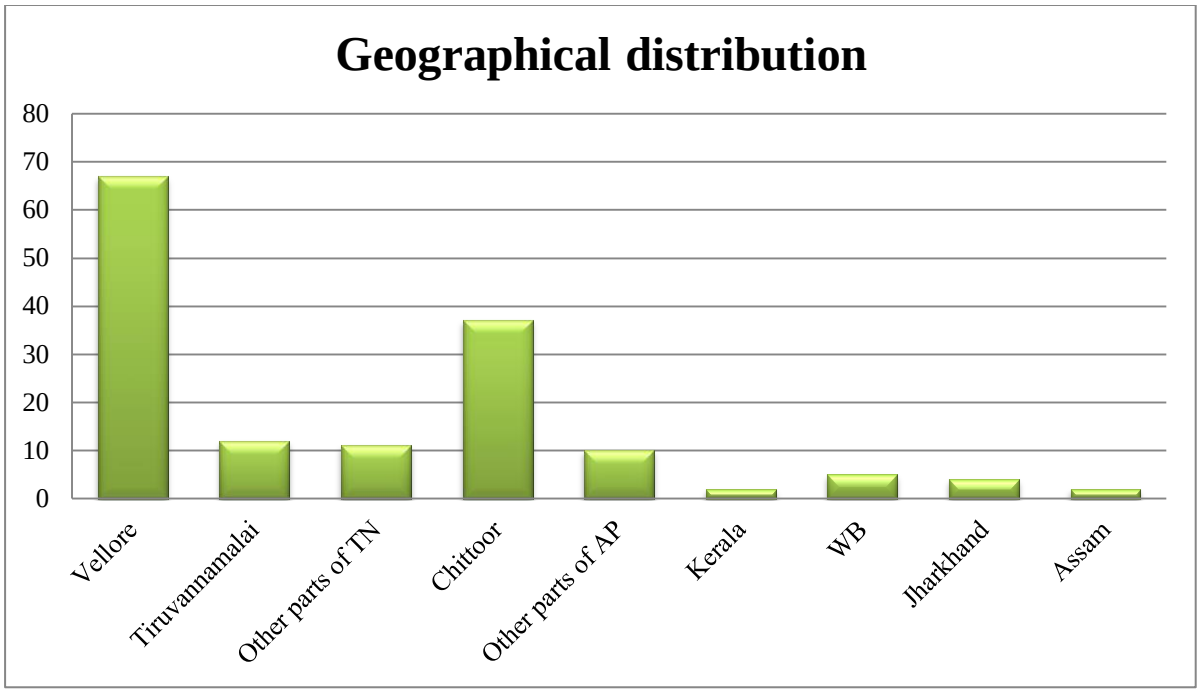


Figure 5.3: Geographical distribution of the patients recruited

Out of the recruited patients 66 (44%) were farmers, followed by 32 (21%) housewives and 18 (12%) professionals (Figure 5.4).

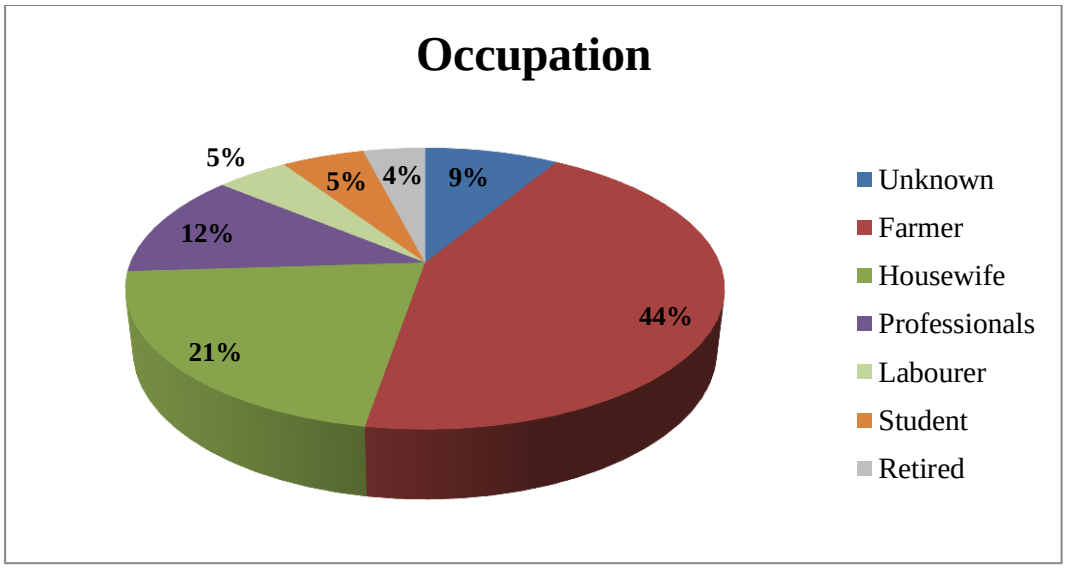


Figure 5.4: Occupation of the study patients

The serum samples of 150 patients recruited in the study were tested for antibodies to leptospira by IgM ELISA (PanBio Ltd), PCR for *rrs* gene for leptospira and LAMP assay for LipL41 and LipL32 genes for leptospira.

Among these patients, totally, 43 were positive for antibodies by IgM ELISA, 10 were positive by LAMP assay and 5 were positive by PCR. Of these, 3 samples were positive by all 3 tests (Figure 5.5).

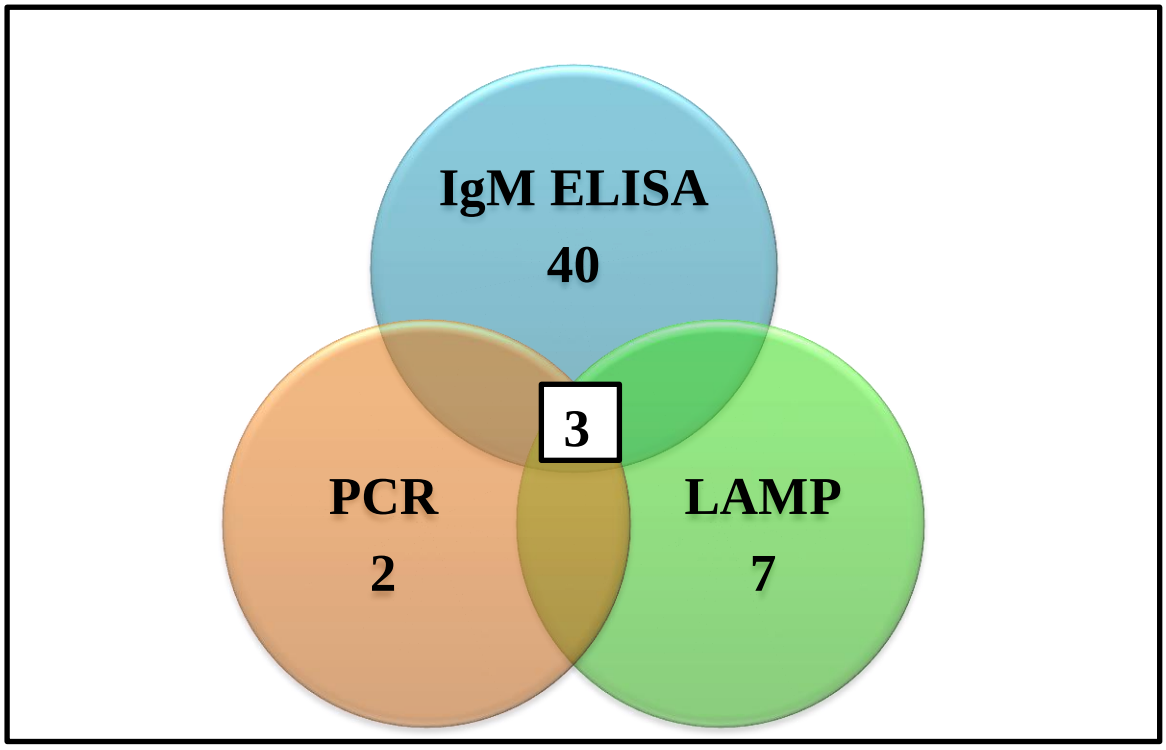


Figure 5.5: Results of three tests used for leptospirosis diagnosis

All these 52 patients fulfilled the case definition for leptospirosis (either positive by a molecular assay or fulfilled modified Faine's criteria).

Among the ELISA positive samples, the values had a range 20 to 54 PanBio units (mean = 25.4, median = 23, mode =21)

Among the samples negative for leptospirosis the ELISA range was 1 to 15 Panbio units (mean = 3.3, median = 3, mode = 2).

Convalescent serum samples were collected from 32 patients after 7 or more days of collection of initial serum and were tested for IgM antibodies to leptospira by ELISA.

Among them, 10 patients continued to be positive for IgM antibodies. However, none seroconverted.

All the patients recruited had fever (≥100º F).

The number of patients with their duration of fever with which they presented to our hospital is shown in Figure 5.6.

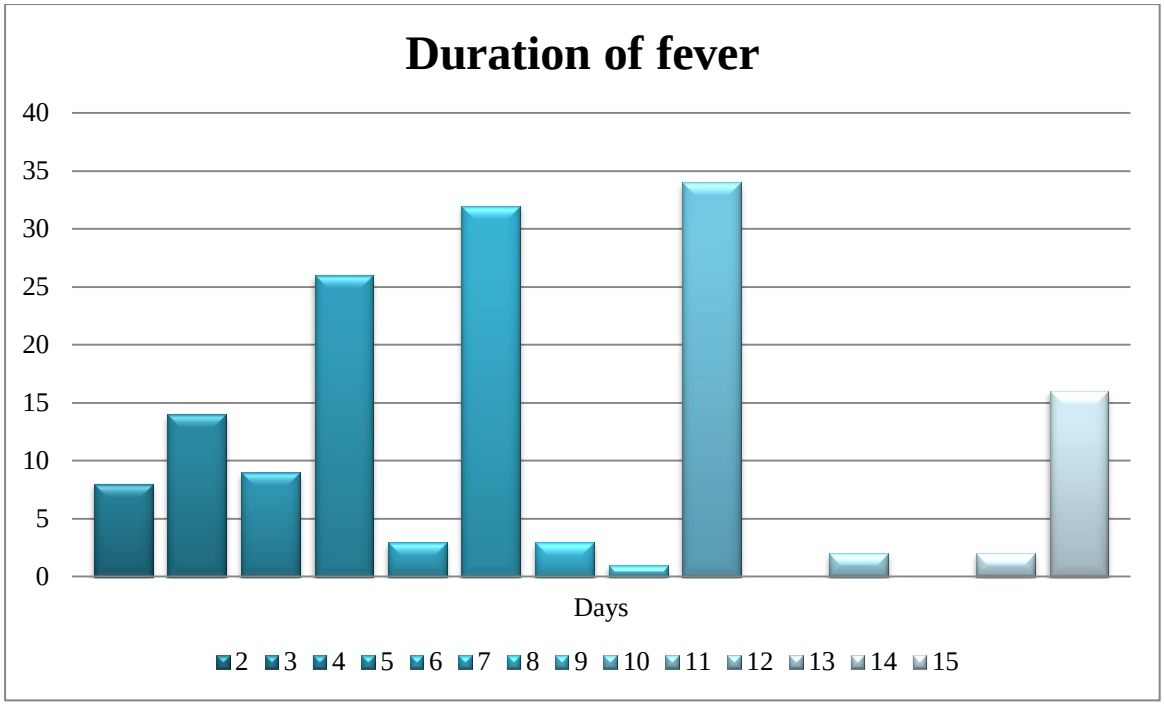


Figure 5.6: Duration of fever in study population

Among these patients, 49(32.67%) had chill and rigor of which 19(36.54%) had leptospirosis while 30(30.61%) did not have leptospirosis (p = 0.461).

The most common symptoms were myalgia, headache, vomiting, and abdominal pain. Among the patients recruited, 60(40%) patients had myalgia, of which 25(48.08%) had leptospirosis and 35(35.71%) were without leptospirosis. (Figure 5.7).

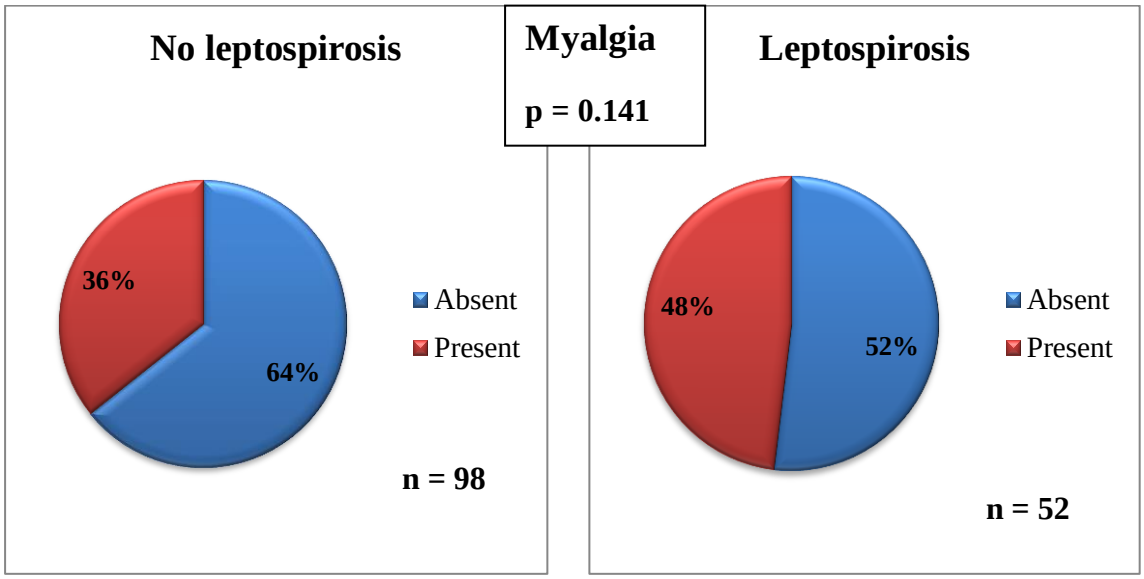


Figure 5.7: Myalgia in patients with and without leptospirosis

Also, 46(30.67%) patients had headache of which 19(36.54%) had leptospirosis and 27(27.55%) did not have leptospirosis (Figure 5.8).

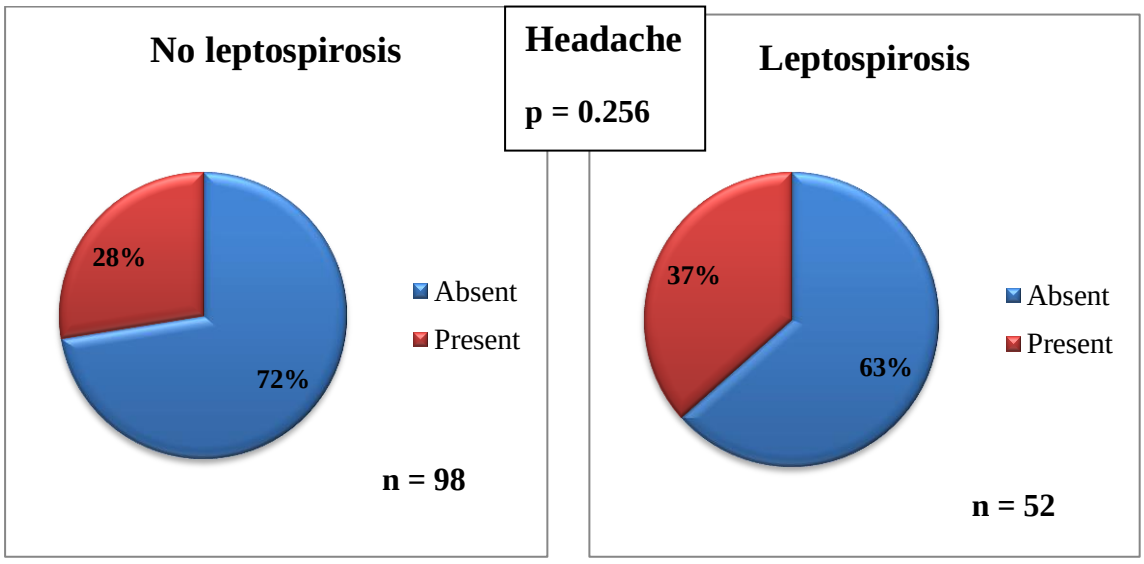


Figure 5.8: Headache in patients with and without leptospirosis

The common gastrointestinal symptoms were abdominal pain in 33(22%), diarrhoea in 21(14%) and vomiting and nausea in 71(47.33%) patients (table 5.3). Abdominal pain was seen in 12(23.08%) patients with leptospirosis and 21(21.43%) patients without leptospirosis. Loose stool was present in 8(15.38%) patients of leptospirosis and 13(13.27%) patients without leptospirosis. Nausea and vomiting was seen in 22(42.31%) leptospirosis patients and 49(50%) of other patients. However, of the leptospirosis patients 40(76.92%) did not have abdominal pain, 44 (84.62%) did not have loose stool and 30(57.69%) did not have nausea or vomiting. Abdominal pain, loose stool and nausea and vomiting were not significantly higher in patients with leptospirosis. Amongst the other patients without leptospira infection, 77(78.57%) did not have abdominal pain, 85(86.73%) did not have loose stool and 49(50%) did not have nausea or vomiting.

Table 5.3: Distribution of gastrointestinal symptoms in patients with and without leptospirosis

Symptoms	Patients without leptospirosis	Patients with leptospirosis	p value
Abdominal pain (n=33)	21(21.43%)	12(23.08%)	0.817
Loose stool (n=21)	13(13.27%)	8(15.38%)	0.722
Nausea and vomiting (n=71)	49(50%)	22(42.31%)	0.369

The common respiratory symptoms were dry cough in 14(9.33%) patients, cough with expectoration in 23(15.44%) patients, respiratory distress in 31(20.67%) patients and chest pain in 1(0.67%) patients. The only patient having chest pain had leptospirosis. Dry cough was seen in 3(5.77%) patients with leptospirosis and 11(11.22%) patients without leptospirosis. Cough with expectoration was present in 12(23.08%) patients of leptospirosis and 11(11.22%) patients without leptospirosis. Respiratory distress was seen in 13(25%) leptospirosis patients and 18(18.37%) of other patients. However, of the leptospirosis patients 49(94.23%) did not have dry cough, 40(76.92%) did not have expectoration and 39(75%) did not have respiratory distress. Cough, expectoration, respiratory distress and chest pain were not significantly higher in patients with leptospirosis. In those without leptospira infection, 87(88.78%) did not have cough, 86(87.75%) did not have expectoration and 80(81.63%) did not have respiratory distress.

Table 5.4: Distribution of respiratory symptoms in patients with and without leptospirosis

Symptoms	Patients without leptospirosis	Patients with leptospirosis	p value
Dry cough (n=14)	11(11.22%)	3(5.77%)	0.274
Cough with expectoration (n=23)	11(11.22%)	12(23.08%)	0.055
Respiratory distress (n=31)	18(18.37%)	13(25%)	0.340
Chest pain (n=1)	0(0%)	1(1.92%)	0.168

In patients with leptospirosis, the urine output was significantly reduced as shown in Figure 5.9. The decreased urine output was seen in 15(28.85%) patients with leptospirosis but in only 13(13.27%) patients without leptospirosis.

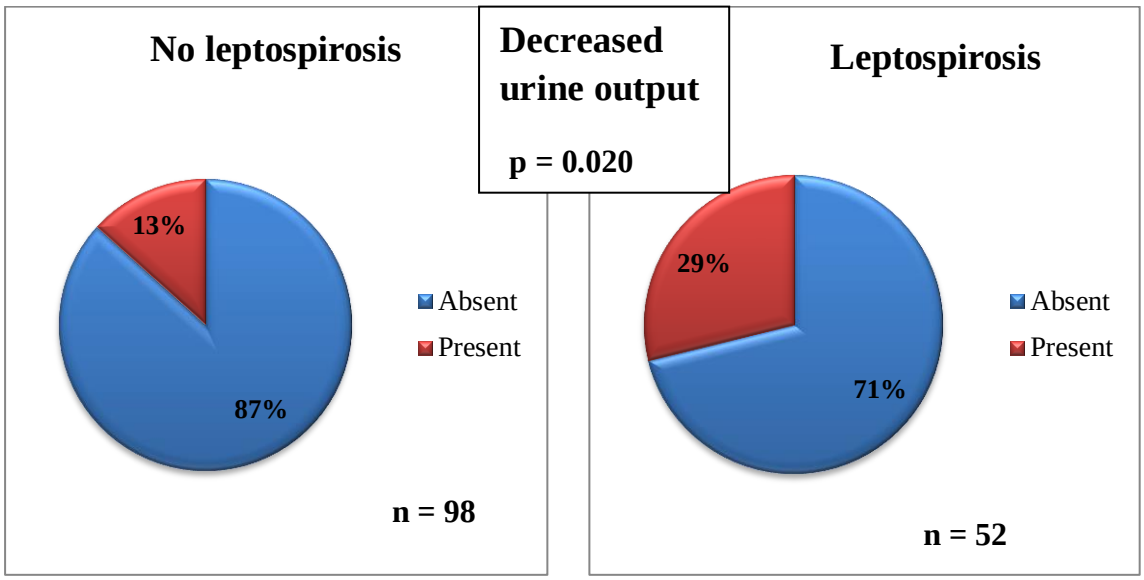


Figure 5.9: Distribution of urine output in the patients with and without leptospirosis

Among the patients recruited, 17(11.33%) had maculopapular rash of which 4(7.69%) had leptospirosis and 13(13.27%) did not have leptospirosis (Figure 5.10).

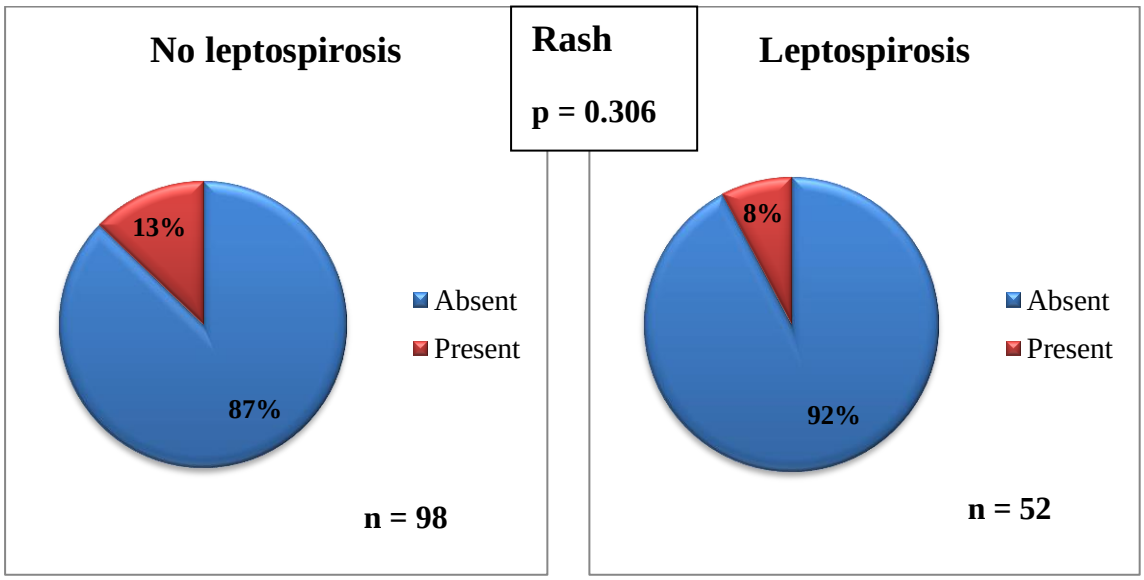


Figure 5.10: Distribution of rash in the patients recruited

The central nervous system involvement was seen in severe disease in 24(16%) patients of which 5(9.62%) had leptospirosis while 19(19.39%) did not have leptospirosis (p = 0.120). The symptoms were seizure 7(5%), altered sensorium 14(9%), delirium 2(1.33%) and diplopia 1(0.67%) (Figure 5.11).

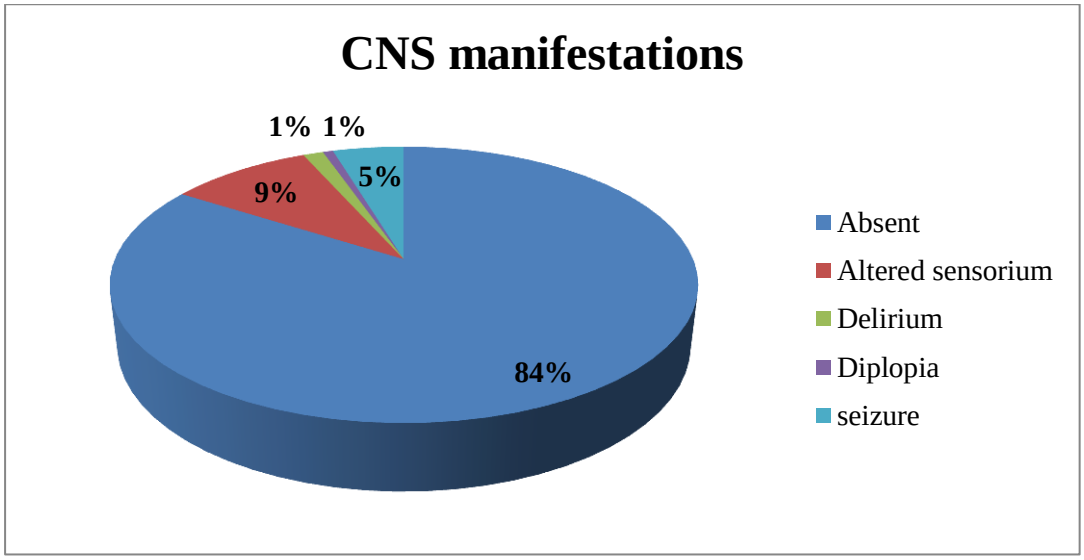


Figure 5.11: Central nervous system manifestations in the patients recruited

Among the patients recruited only 13(8.67%) had bleeding manifestations (Figure 5.12) of which 4(7.69%) had leptospirosis and 9(9.18%) did not have leptospirosis (p = 0.757).

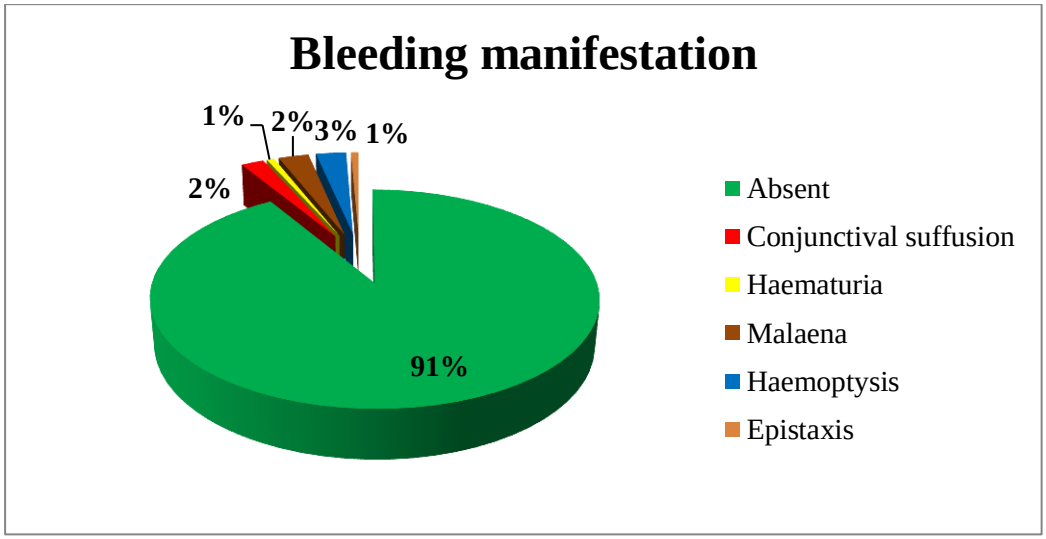


Figure 5.12: Bleeding manifestations in the study patients

Among the patients who had leptospirosis 31(59.61%) had jaundice with increased bilirubin (>1mg/dl) and 21(40.38%) had normal bilirubin (≤1mg/dl). Among the patients who did not have leptospirosis 56(57.14%) had jaundice while 42(42.85%) did not have jaundice. Hence, presence of jaundice is not significant in leptospirosis patients (p = 0.052) (table 5.5)

Table 5.5: Bilirubin in patients with and without leptospirosis

Bilirubin	Without leptospirosis	Leptospirosis
Bilirubin ≤1mg/dl	42	21
Bilirubin >1-3mg/dl	26	6
Bilirubin >3-10mg/dl	25	16
Bilirubin >10mg/dl	5	9
Total	98	52

In patients with leptospirosis only 6(20%) had bilirubin 1-3mg/dl while 25(48%) had moderate/severe (>3mg/dl bilirubin) jaundice while in patients without leptospirosis 26(26.5%) had mild and 31(31.6%) had moderates/severe jaundice (Figure 5.13). Severe jaundice is significantly related to leptospirosis.

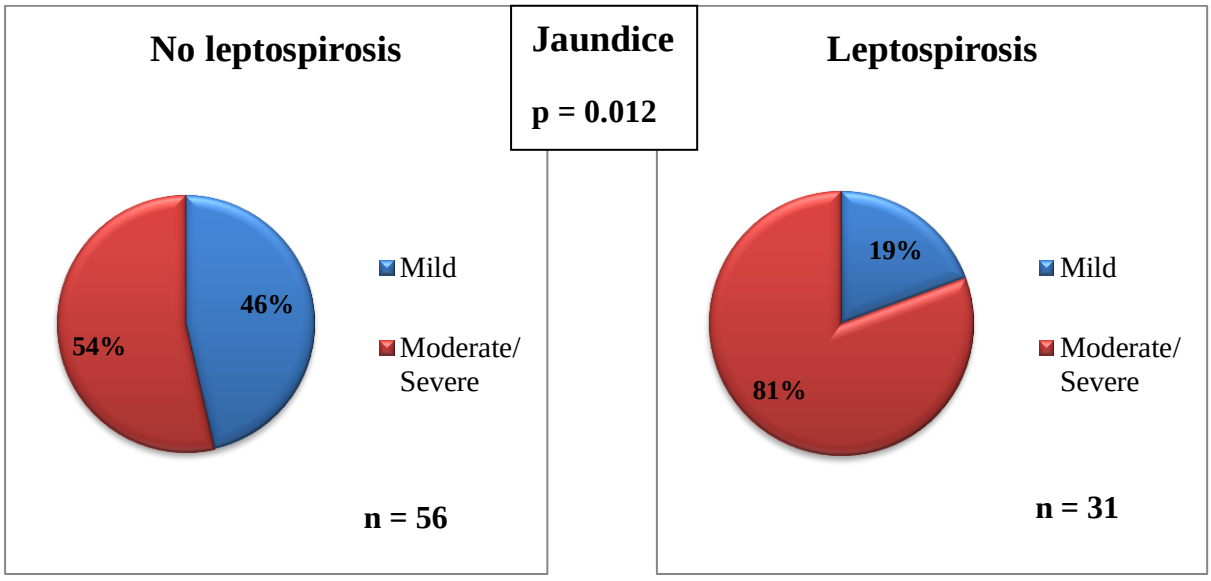


Figure 5.13: Severity of jaundice in patients of leptospirosis

In the patients recruited, 56(37.3%) had raised creatinine level (>1.4mg/dl). Renal damage as indicated by raised creatinine level is not significantly higher in patients with leptospirosis as 22(42.3%) patients with leptospirosis had renal damage whereas 34(34.69%) patients had renal damage without leptospirosis as shown in table 5.6 (P = 0.243)

Table 5.6: Creatinine levels in patients recruited

Creatinine	Without leptospirosis	Leptospirosis
Creatinine ≤1.4mg/dl	64	30
Creatinine >1.4-2.5mg/dl	11	2
Creatinine >2.5-4mg/dl	10	7
Creatinine >4 mg/dl	13	13
Total	98	52

There were 20 patients with leptospirosis who had creatinine >2.5mg/dl whereas 23 patients without leptospirosis had creatinine >2.5mg/dl as compared to 2 patients of leptospirosis and 11 patients of other diseases with creatinine >1.4-2.5mg/dl (Figure 5.14)

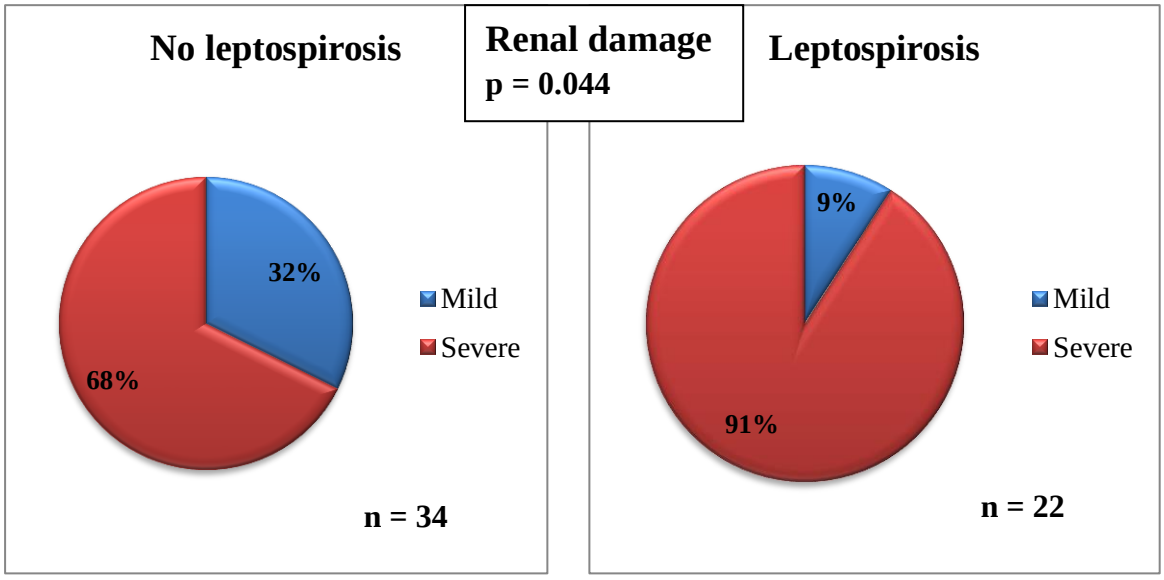


Figure 5.14: Severity of renal damage in patients with leptospirosis

Among the patients 31(20.67%) patients had acute respiratory distress syndrome, of which 13(25%) had leptospirosis and 18(18.37%) did not have leptospirosis (Figure 5.15)

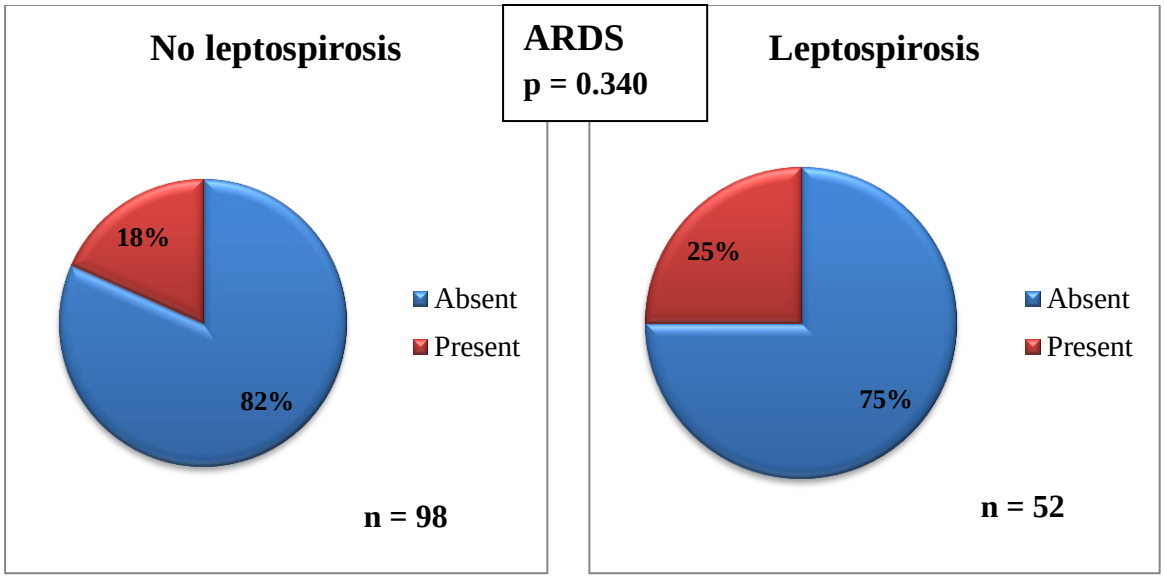


Figure 5.15: Acute respiratory distress syndrome in study population

In the study population, 97(64.6%) had a fall in the blood platelet count (<150000/mm^3 blood). It was reduced in 40(76.92%) patients with leptospirosis and 57(58.16%) patients without leptospirosis (Figure 5.16)

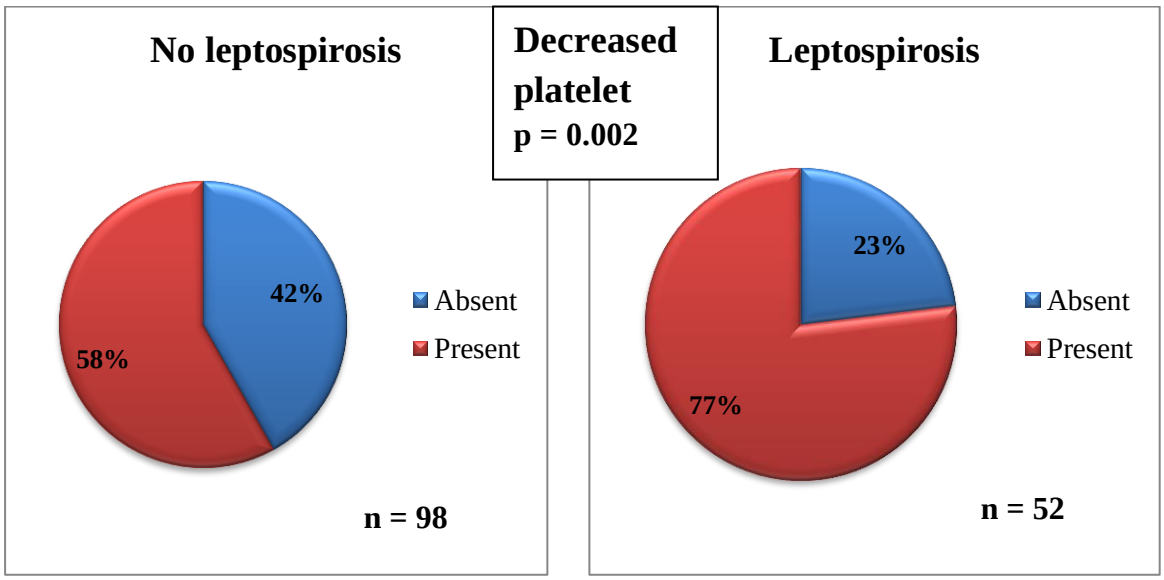


Figure 5.16: Platelet count of the patients recruited

A nested PCR was done for the detection of *rrs* gene of leptospira. The resulting amplicon is 547bp.

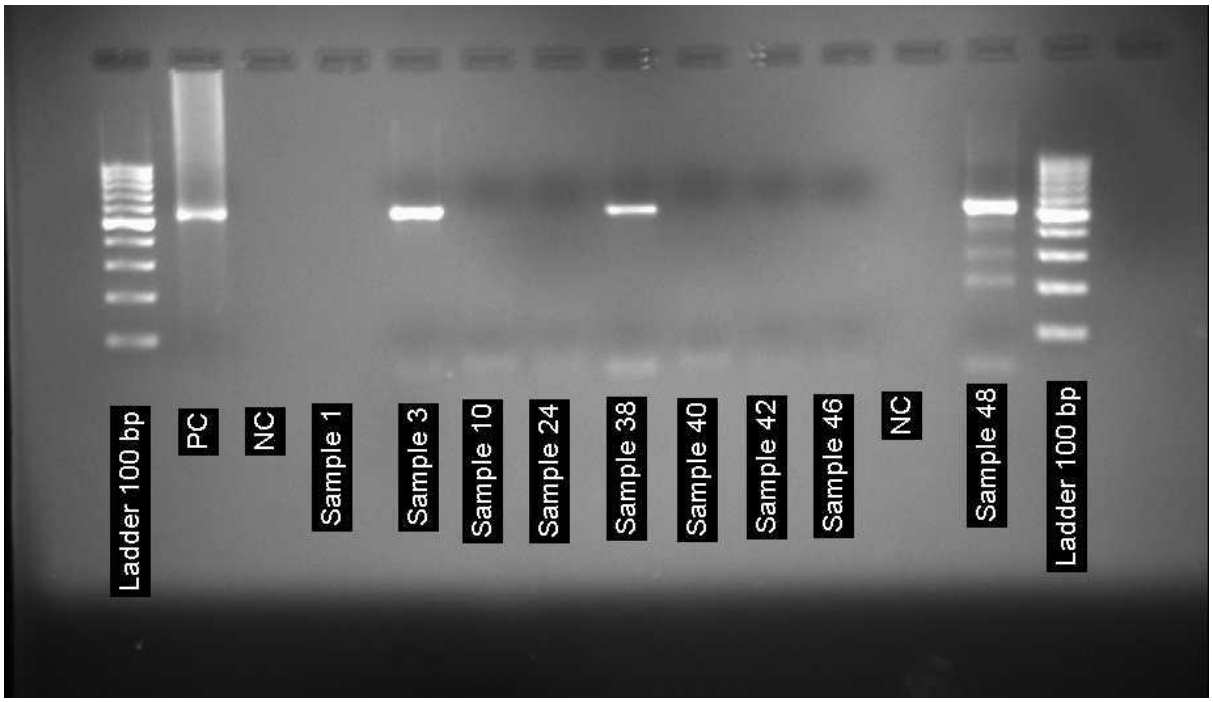


Figure 5.17: Gel picture of the PCR for leptospirosis

A ladder of 100bp was used as a reference for calculation of the base pair. The positive control (PC) was the control strain of *Leptospira interrogans* serovar Icterohaemorrhagiae, which showed a band at 547bp for *rrs* gene. The negative control (NC) did not show any band. As both the controls were satisfactory, the readings for the samples were taken.

The sample 3, sample 38 and sample 48 showed bands at 547bp and were considered positive for *rrs* gene for leptospira.

The sample 1, sample 10, sample 24, sample 40, sample 42, sample 46 had no bands and were taken as negative for leptospirosis.

A loop – mediated isothermal amplification assay (LAMP) was done for detection of LipL32 and LipL41 genes of leptospira.

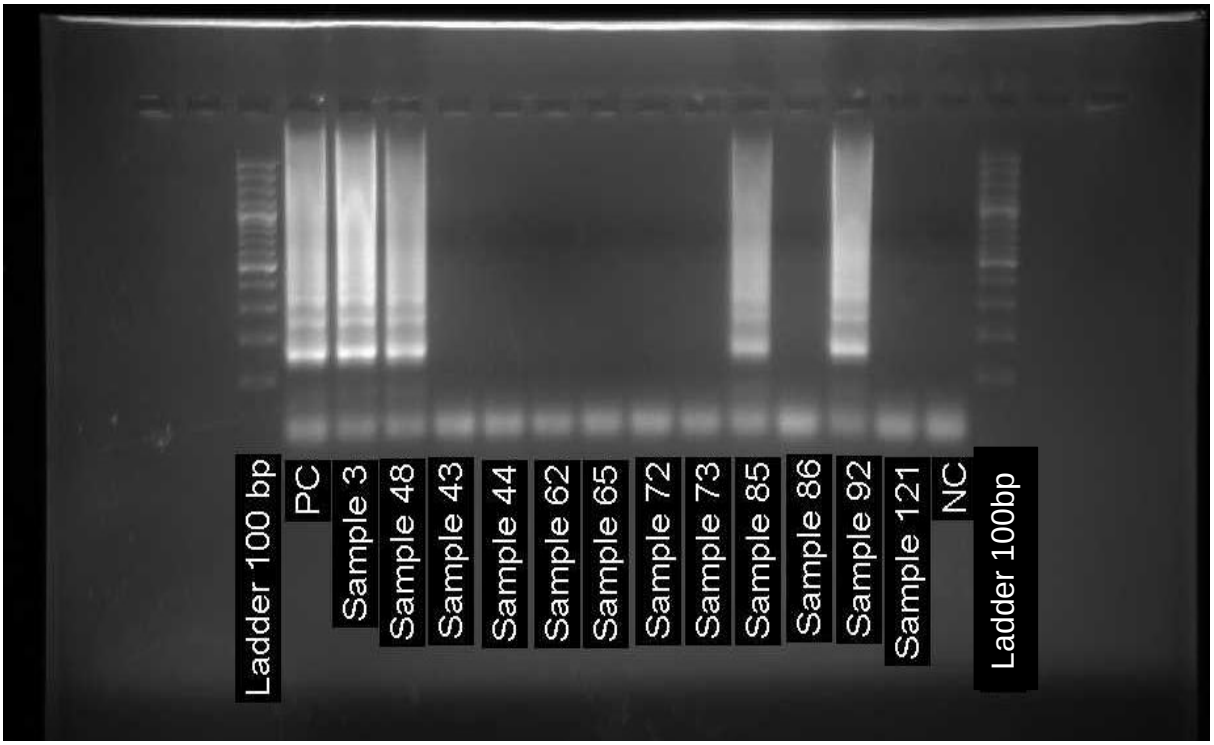


Figure 5.18: Gel picture of the LAMP assay

A ladder of 100bp was used as a reference for calculation of the base pair. The positive control (PC) was the control strain of *Leptospira interrogans* serovar Icterohaemorrhagiae, which showed a LAMP band. The negative control (NC) did not show any band. As both the controls were satisfactory, the readings for the samples were taken. For the samples, the reading were taken by comparing it with the banding pattern of the control strain.

The sample 3, sample 48, sample 85, sample 92 had bands similar to the control strain band and were taken as positive by LAMP assay. The samples 43, sample 44, sample 62, sample 65, sample 72, sample 73, sample 86 and sample 121 did not have any band and were taken as negative by LAMP assay.

Evaluation of the three tests

By using latent class analysis for the three tests ELISA, PCR and LAMP for diagnosis of leptospirosis it was found that there is a prevalence of 2% of leptospirosis among the patients recruited.

Table 5.7: Result of diagnostic assay

ELISA	PCR	LAMP	Positives
+	+	+	3
+	-	-	40
-	+	-	2
-	-	+	7

As the prevalence is very low, the sensitivity of the tests show that all the three tests are highly sensitive (table 9).However, the specificity is highest for PCR with 98.64%, followed by LAMP 95.24% and least for ELISA 72.79% as shown in table 5.8.

Table 5.8: Performance of the tests for diagnosis of leptospirosis

	Test	
Sensitivity	ELISA	100%
	PCR	100%
	LAMP	100%
Specificity	ELISA	72.79%
	PCR	98.64%
	LAMP	95.24%

So, it is evident from the table that LAMP assay has specificity very close to that of PCR.

Utility of LAMP assay

It was seen that LAMP assay was positive for all 10 (100%) within the first week of illness. In case of PCR 3(60%) were positive in the first week of illness and other 2(40%) on 10th day of fever. This is shown in Figure 5.19. By ELISA IgM antibodies were detectable within 7 days of illness for 20(46.5%) and 23(53.4%) were positive between 8-15 days of fever.

This shows that LAMP assay is a reliable test in the first week of illness and IgM ELISA is useful in the second week of fever.

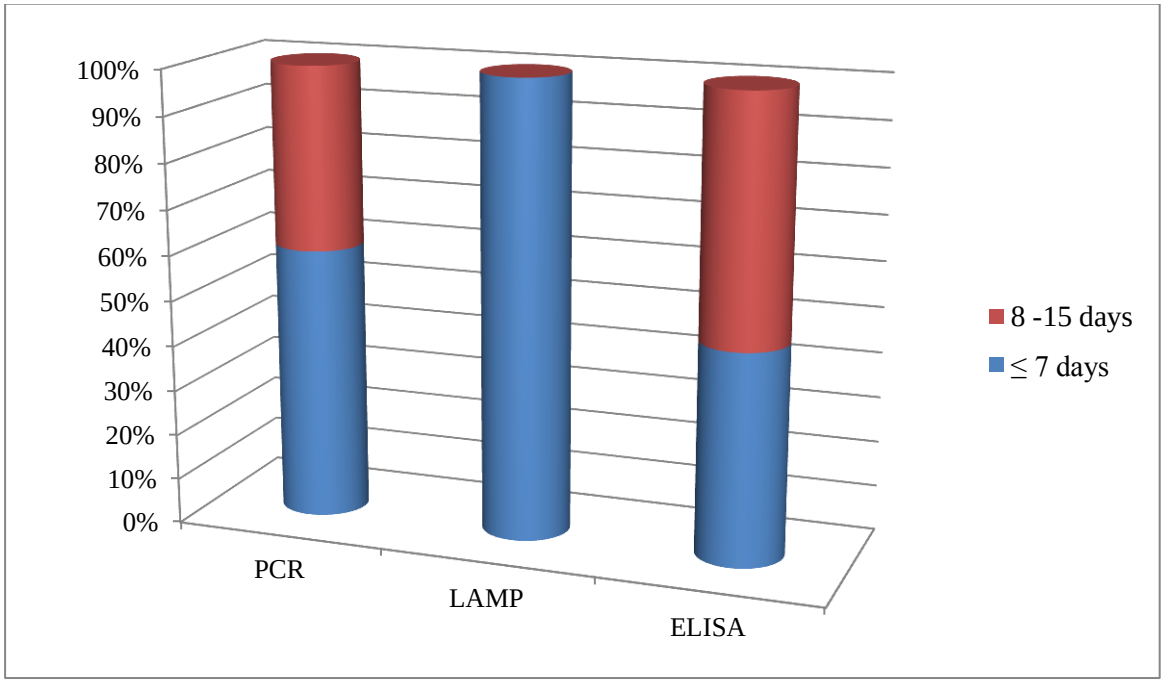


Figure 5.19: Relation between duration of fever and leptospira assay performance

Sequencing was done for sample 38 and 48. The sequence of sample 38 is shown in Figure 5.20 below.

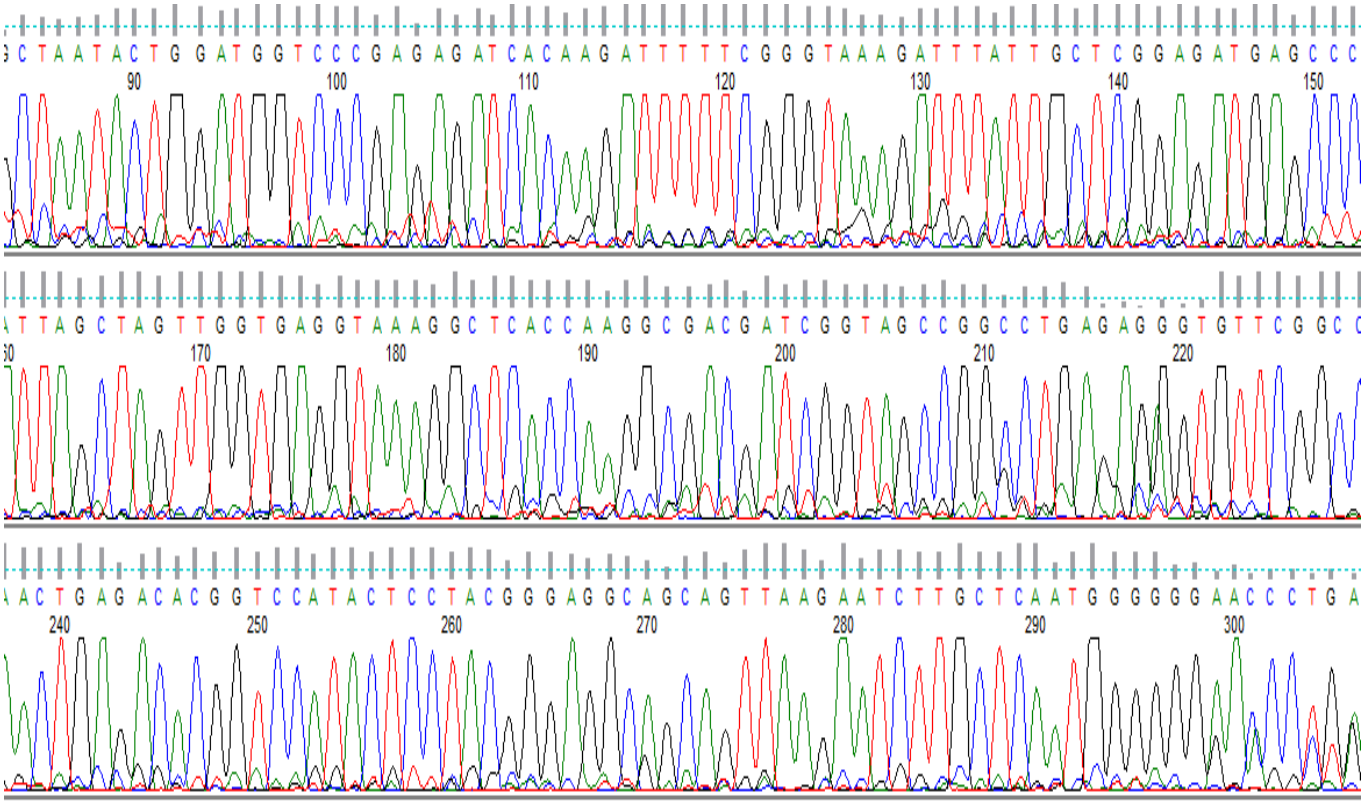


Figure 5.20: Genomic sequence of sample 38

The identity of the two samples sequenced after analysis with the available sequences in genebank are given below in table 5.9

Table 5.9: Identity of the sequence of the two isolates

Identity with the strain	Sample 38 538 nucleotides	Sample 48 546 nucleotides
Leptospira interrogans serovar Canicola	98%	97%
Leptospira interrogans serovar Hebdomadis		
Leptospira interrogans serovar Zanoni		
Leptospira interrogans serovar Hardjo		
Leptospira interrogans serovar Bratislava		
Leptospira interrogans serovar Grippotyphosa		
Leptospira interrogans serovar Australis		96%
Leptospira interrogans serovar Kremastos		
Leptospira interrogans serovar Pomona strain Pomona		
Leptospira interrogans serovar Lai		
Leptospira interrogans strain Icterohaemorrhagiae		
Leptospira interrogans strain Coppenhageni		
Leptospira interrogans serovar Hardjo-prajitno		
Leptospira kirschneri strain Grippotyphosa		
Uncultured leptospira		

Among the patients recruited, 52(34.7%) had evidence of leptospira infection and 56(37.3%) had IgM antibodies to *Orientia tsutsugamushi* and 22 patients had IgM antibodies to both leptospira and scrub typhus (Figure 5.21).

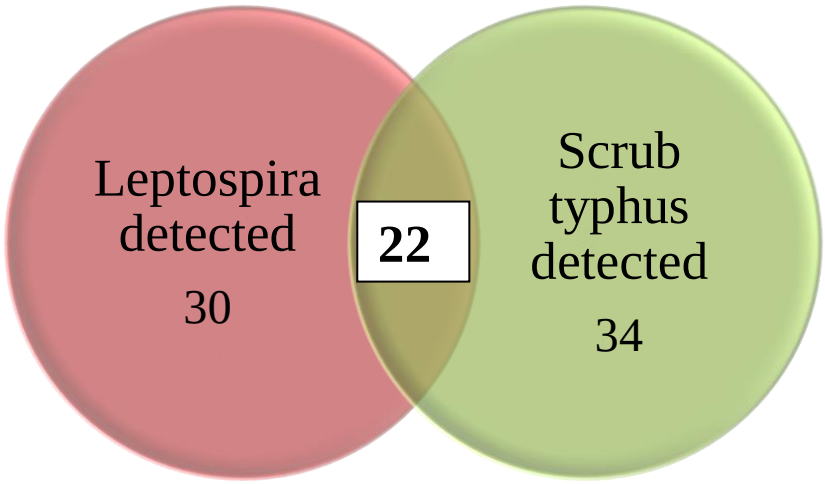


Figure 5.21: Samples positive for leptospirosis and scrub typhus

Most of the patients improved with treatment with doxycycline, ceftriaxone, azithromycin or a combinations of these drugs (Figure 5.22)

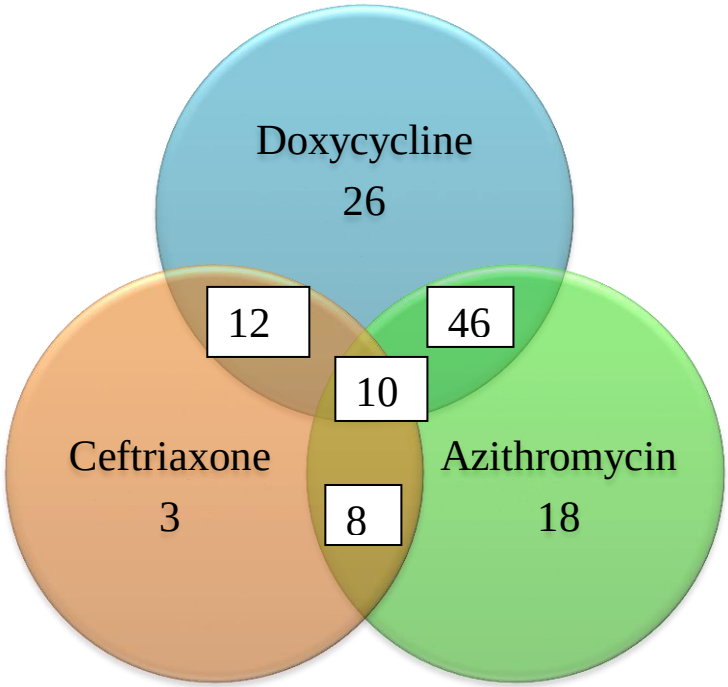


Figure 5.22: Antimicrobials received by the patients recruited

All the patients who were positive for scrub typhus or leptospira by any of the tests received either or in combinations of the three antimicrobials – doxycycline, ceftriaxone, azithromycin except one patient who received meropenem and improved in 25 days.

Of the 150 patients recruited in the study, only 7(4.6%) succumbed to the illness. Of these three patients were diagnosed as leptospirosis by modified Faine's criteria.

Table 5.10: Details of patients who succumbed to illness

Patient no	Clinical diagnosis	Comment
A	Leptospirosis	Died within 6 hours
B	Scrub typhus	Died in 48 hours
C	Acute febrile illness	Died within 24 hours
D	Tuberculous meningitis	Died in 7 days
E	Ventilator associated pneumonia	Died in 10 days
F	Septic shock	Secondary infection
G	Acute febrile illness	Died within 24 hours

Discussion

Leptospirosis is a common cause of acute febrile illness especially during the monsoon months. As the clinical features are non-specific the differentials considered are malaria, dengue, scrub typhus, rickettsial fever and typhoid fever (1). Hence, laboratory diagnosis of leptospirosis is essential for confirmation of the disease. The gold standard tests for diagnosis of leptospirosis are culture and microscopic agglutination test (MAT) which are not feasible as routine diagnostic tests (1). Currently the most widely used method for diagnosis of leptospirosis is IgM antibody detection by ELISA. In addition, molecular tests are considered as good as the reference test for diagnosis (114).

This pilot study was done to evaluate the utility of IgM ELISA, PCR and LAMP assay in the diagnosis of leptospirosis. A total of 150 patients who fulfilled the inclusion and exclusion criteria were recruited. Among the patients enrolled, 52 were taken as cases of leptospirosis based on the case definition adopted for this study. These included three samples positive by all three tests (PCR, LAMP and IgM ELISA), two samples positive by PCR for *rrs* gene and seven samples positive by LAMP assay for LipL32 and LipL41 genes and IgM antibodies to leptospira detected by ELISA in sera from 40 patients who fulfilled modified Faine's criteria. Fidelity of amplification of leptospira specific DNA was confirmed by sequencing the *rrs* gene product of two samples which were positive by PCR and LAMP assay.

Validation of ELISA

It is recommended that cut off values for infectious disease diagnosis using serological assay like ELISA should be arrived at by testing sera from healthy individuals, those with proven disease and also others with clinically similar illness. The usage of well characterized archived specimens, consisting of the aforementioned groups allows rapid assessment of the clinical utility of the new assay (16). It is to be noted that if the cut-off is too low it will over-estimate whereas if the cut-off is too high it will under-estimate the burden of disease in the given community or region.

The prevalence of antibodies in the plasma samples of healthy blood donors was determined in this study. The plasma and serum samples of 20 healthy donors showed similar results, therefore, it was concluded that plasma values could be extrapolated to serum. Serum samples of patients who present with an illness like scrub typhus, sepsis, enteric fever, malaria and dengue (clinically indistinguishable from leptospirosis) were used to determine and validate the ELISA cut-off.

Validation of a diagnostic cut-off for a serological assay is necessary in countries, especially South Asian and South-East Asian nations. This is because infections are a major cause of morbidity and mortality in these regions and is supported by evidence provided by various studies. In a study undertaken by Desakorn V *et al* from Thailand the PanBio IgM ELISA was evaluated by testing sera from healthy blood donors. Using the manufacturer's cut-off, which is 11 PanBio units, the sensitivity and specificity of the ELISA on paired sera was 90.8% and 55.1% respectively. When the cut-off was placed at 20 PanBio units, the sensitivity fell to76.1% whereas the specificity improved to 82.6% (115). In another study done by Tanganuchitcharnchai A *et al*, using a cut-off of OD $\geq$ 0.75 for IgM antibodies by ELISA (Standard Diagnostics, Inc, Gyeonggi-do, Republic of Korea), a sensitivity of 95%

and specificity of 41% was observed in normal sera. On validation of the ELISA, using serum samples of patients with fever, the cut-off was found to be at OD of 1.7, the sensitivity decreased to 70% and specificity increased to 78% (116). The results of these two studies prove that a cut–off for serological diagnosis of leptospirosis has to be determined prior to its deployment for routine identification of cases.

In this study the diagnostic cut-off for the Leptospira PanBio IgM ELISA (PanBio Ltd, Brisbane, Australia) was determined to be ≥20 PanBio units. The other IgM ELSA for leptospira like Virion serion (Serion Immunodiagnostics, Wurzburg, Germany) and InBios (InBios International, Seattle, WA, USA) were also validated and an OD ≥0.7 and ≥1.0 were considered significant. The three different ELISA kits evaluated had three different cut-off values for the detection of IgM antibodies to leptospira. This demonstrates that the cut-off obtained for one kit cannot be used for another kit though it detects the same parameter.

The cut-off for detection of IgG antibodies to leptospira (Virion serion, Serion Immunodiagnostics, Wurzburg, Germany) was high (OD ≥2.7). This may be due to high seroprevalence but this could not be confirmed as only one assay for detection of IgG antibodies to leptospira could be performed on samples used for validation. In addition, the cut off OD for scrub typhus IgM ELISA (InBios International, Seattle, WA, USA) was found to be ≥0.5 which was the same as previously described (117).

Relation of clinical features of leptospirosis

According to World Health Organisation, the case definition of leptospirosis includes an acute febrile illness with headache, myalgia, conjunctival suffusion, anuria/oliguria, jaundice, cough, haemoptysis, breathlessness, haemorrhage, rash, nausea, vomiting, abdominal pain,

diarrhoea and meningeal irritation (44). The above mentioned clinical features were present in many of the patients recruited. However, only oliguria, moderate/severe jaundice and renal damage were significantly related to the patients with leptospirosis in the present study (p value <0.05) and only 7 patients out of which 3 had leptospirosis succumbed to the illness.

In this study among the leptospirosis patients, in addition to fever, 48.08% had myalgia, 36.54% had headache, 28.85% had oliguria, 59.61% had raised bilirubin and 42.3% had renal compromise as evidenced by raised creatinine level. Other studies performed in India have noted similar clinical findings in patients with leptospirosis. Datta *et al* observed that myalgia was seen in 78.4% and icterus in 74.5%, headache in 41.2% and oliguria in 29.4% of leptospirosis patients (66). Chaudhry *et al* reported that the common clinical features were vomiting/nausea (49.4%), headache (50.5%), myalgia (52.8%), renal involvement (54%) and raised bilirubin (59.7%) (36). Using Modified Faine's criteria Debmandal *et al* found that the commonest features were headache (100%) and jaundice (93.92%), 25.23% leptospirosis cases had increased bilirubin (34).

Modified Faine's criteria for diagnosis of leptospirosis

In a leptospira study, Chauhan and co-workers, demonstrated that out of 13 cases which were confirmed as leptospirosis by ELISA or PCR, all fulfilled modified Faine's criteria though only 7 fulfilled Faine's criteria (118). This shows that Modified Faine's criteria is more suitable for Indian scenario. The reason for modification of Faine's criteria was that rainfall should be taken into account as most outbreaks of leptospirosis occur during monsoon. The microscopic agglutination test (MAT) is the serological reference standard but it is complicated and difficult test to perform. Hence, ELISA and slide agglutination test (SAT) which are simple and easy to perform have also been included in the criteria (109). In

this study all 43 samples which were IgM ELISA positive fulfilled modified Faine's criteria, including those who were scrub typhus IgM ELISA positive. The Faine's criteria was not applied as microscopic agglutination test or culture could not be performed.

Evaluation of tests for diagnosis of leptospirosis

In this pilot study the prevalence of leptospirosis was found to be low (2%), the sensitivity of IgM ELISA, LAMP and PCR for diagnosis of leptospirosis was 100% by latent class analysis (LCA). The specificity of PCR for the target *rrs* gene (547bp) was found to be 98.64% , that of LAMP assay was 95.24% using targets LipL32 and LipL41 gene whereas the specificity of ELISA was 72.79%. In other studies targeting the leptospira *rrs* gene the specificity was found to be between 90-100%. The sensitivity varied between 56-94.4% (8,101–103). The LAMP assay for leptospirosis has been evaluated by only four research groups. In a study done by Lin X *et al* the detection limit was same as PCR (100 genome equivalents) the target being LipL41 gene (13). Sonthayanon *et al* reported that with the target *rrs* gene and LipL41 gene, the sensitivity was 43.6% and 37.6% and specificity was 83.5% and 90.2% respectively for the two targets (6). So, in comparison, this study also had a similar specificity. In another study conducted by Koizumi *et al*, the specificity of LAMP assay with *rrs* gene as target was 66.7% (14). From the last two studies it is evident that LipL41 has higher specificity compared to *rrs* gene for detection of leptospira DNA by LAMP assay. In the present study the LAMP assay had a high specificity as it had two targets LipL32 and LipL41. This explains the reason of it picking up the samples which were neither picked up by PCR nor ELISA. Interestingly, there were two samples which were positive for *rrs* gene by PCR but not picked up by LAMP assay or IgM detection by ELISA. This may be due to the fact that pathogenic leptospira have two copies of *rrs* but a single copy of lipL32 gene (8).

All 10 samples positive by leptospira LAMP assay occurred in individuals whose duration of illness did not exceed one week. This suggests that LAMP assay is more likely to be positive in those with fever less than 7 days. The leptospira LAMP assay being negative for patients in whom illness exceeded more than a week may be due to over the counter use of antibiotics and disappearance or decrease in leptospira levels in blood in the second week of illness. In those with leptospirosis, IgM antibody levels classically start rising in the middle of first week and peak by 7^{th} day of illness (1). In this study, the IgM antibodies were detectable from 3^{rd} day of fever and the diagnosis of leptospirosis was heavily dependent on detection of IgM antibodies in the second week. This confirms the finding that leptospira cannot be detected in blood by culture or nucleic acid amplification tests like PCR or LAMP after the first week of illness (14). As already described, the sensitivity of a properly evaluated serological assay like ELISA is high (16). So, it is a useful tool for diagnosis of leptospirosis in a tertiary care centre like ours. This is of great importance as most of these patients are referred to our hospital in the second week of illness and have not responded to treatment at the peripheral centres.

Serological confirmation of a diagnosis by ELISA involves demonstration of seroconversion by testing for IgM and IgG antibodies. Seroconversion is said to occur when either significant IgG antibodies are demonstrable in convalescent sera and/ or IgM antibodies decline below the diagnostic threshold (119). In spite of best efforts, convalescent sera could be obtained from only 32 of the 150 patients (7 or more days after collection of the initial sample). Amongst these patients whoever had detectable antibodies in the initial serum samples (10 of 32) continued to have IgM antibodies in the convalescent serum as detected by ELISA. As the IgG ELISA on paired sera could not be performed, seroconversion in the 32 patients for whom the same was available could not be demonstrated.

Co-infection

Amongst the 43 cases of leptospirosis according to modified Faine's criteria, 22 had IgM antibodies to *O. tsustsugamushi.* Of these 22 patients, one patient was positive by leptospira PCR and LAMP whereas another was positive by LAMP assay only. These 22 patients could be possible cases of co-infection or cross reaction. In a study done by Sonthayanon *et al,* 82 cases with serological evidence of scrub typhus (by IFA) and leptospirosis (by MAT) were tested by duplex PCR targeting the 16S rRNA gene of both leptospira and *Orientia tsutsugamushi* to confirm or rule out co-infection. Scrub typhus and leptospira specific nucleic acid was detected in 6% (5/82), whereas DNA specific only for scrub typhus was detected in 5% (4/82) and for only leptopsira in 46% (38/82) (71). This demonstrates that molecular evidence alone cannot be used to confirm or eliminate co-infection. This is especially true when samples are tested later in the course of the illness as from second week onwards serological assays are more likely to be positive. In the 22 samples with serological evidence of co-infection with scrub typhus and leptospirosis around 75% had fever of duration more than 7 days. Paired sera (acute and convalescent serum) was available for 2 of these patients and both showed diagnostically significant levels of IgM antibodies to scrub typhus and leptospira respectively by ELISA in paired sera. As, molecular confirmation of scrub typhus was beyond the scope of this study and paired sera was not available and even if available could not be tested for IgG antibodies, the issue of co-infection could not be resolved in any of these patients. However, occurance of co-infection with scrub typhus and leptospirosis had been demonstrated by many research groups using serology (68–70). IgM antibodies to scrub typhus in the presence of an eschar combined with a dramatic response to fever is considered confirmation of scrub typhus (117). As per the study criteria, only those patients who did not have pathognomonic eschar were evaluated.

Limitations of the study

The limitations of the study were that IgG antibody testing could not be done on serum samples of patients with suspicion of leptospirosis. Convalescent sera could be collected only in 32 patients. This is attributed to the fact that the patients who responded to the treatment seldom return for follow-up. Molecular assays for scrub typhus could not be performed on the serum samples which would have been helpful in resolving the problem of co-infection.

To summarise, a prospective study was conducted to compare serological and molecular assays for diagnosis of leptospirosis. The findings suggest that molecular assays are more likely to be positive in the first week of illness whereas in the second week serological assays are the mainstay of diagnosis. These preliminary findings need to be verified by larger community based prospective studies using more specific assays for leptospira diagnosis.

Summary and conclusion

A pilot study was undertaken to prospectively evaluate the efficacy of a serological assay (IgM ELISA) and molecular techniques (PCR and LAMP assay) for diagnosis of leptospirosis in 150 clinically suspected cases based on inclusion and exclusion criteria.

1. The ELISA for detection of IgM antibodies to scrub typhus was validated and a diagnostic cut-off was determined using samples from healthy blood donors and patients with scrub typhus, sepsis, enteric fever, malaria and dengue.
2. A nested PCR to detect *rrs* gene (547bp) of leptospira and loop mediated isothermal amplification (LAMP) assay to detect LipL32 and LipL41 genes of leptospira and IgM antibody detection by ELISA was standardised and performed.
3. Using the case definition of leptospirosis (positive by molecular assays or those fulfilling the modified Faine's criteria) 52 patients were diagnosed as cases of leptospirosis.
4. Urine output and platelet count was significantly reduced in patients with leptospirosis ($p<0.05$). Severe jaundice and renal compromise as shown by raised bilirubin and creatinine levels were significantly related to leptospirosis ($p<0.05$).
5. Among the leptospirosis cases, 3 samples were positive by all the three tests, 2 samples were positive only by PCR, 7 samples were positive only by LAMP assay and in 40 samples only IgM antibodies to leptospira were detected by ELISA.
6. Using latent class analysis, PCR had a specificity of 98.64%, LAMP assay had a specificity of 95.24% and IgM ELISA had a specificity of 72.79% though all the three tests had a sensitivity of 100%.

7. Sequencing of two amplified products of PCR which were also LAMP positive confirmed the fidelity of amplification of *Leptospira interrrogans.*
8. In the early phase of illness (within 7 days) LAMP assay performed better for diagnosis of leptospirosis whereas IgM ELISA was the mainstay of diagnosis from second week onwards.
9. Among the leptospirosis patients, 22 had IgM antibodies to scrub typhus as detected by ELISA. It was not known whether these were cross reaction or co-infection with scrub typhus.
10. The findings in this study need to be prospectively evaluated further in community based studies using more specific tools for confirming leptospirosis with an emphasis on testing paired serum samples.

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Annexure I

PATIENT INFORMATION SHEET

Leptospirosis is a disease caused by a germ and is a widespread zoonotic disease (an animal disease which can also affect humans). Humans acquire this infection when they come in direct or indirect contact with urine of an infected animal. The infection can present as mild disease or very severe disease. In those with severe disease, there can be jaundice and organ damage in addition to the fever which is associated with a high death rate. Rapid and correct diagnosis is of utmost importance for starting appropriate treatment which will prevent severe illness and death.

This study titled "Utility of loop-mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis: A pilot study" is aimed at finding out the usefulness of the currently used laboratory test (ELISA) and a new test (LAMP) for the diagnosis of this disease.

You are requested to participate in this study. Your identity and the results of the tests will remain confidential.

For the study, a blood sample will be collected at the time of your admission again 7 days after the first sample. There is no foreseeable risk (risk of injury is very minimal) in participation in the study as the sample being asked for, is routinely obtained on most patients as part of the standard testing protocol for detection or confirmation of the cause of ill-health.

The result of the tests will be made known to you, if you consent to being informed. No other monetary or material initiative is provided for participation in the study. The tests are conducted free of charge and there is no provision for compensation for study related injury.

Participation is voluntary and not mandatory. You can withdraw from the study at any time. Refusal to participate will not involve any penalty or loss of benefit to which you are otherwise entitled.

Kindly ask questions and clear doubts before participation as some of the medical terms may not be familiar to you.

Your participation is important and of immense value as the results of this study will be useful in determining the utility of these tests for the rapid and correct diagnosis of this infection which can be life threatening especially in those who are very sick.

Contact: Mallika Sengupta
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Christian Medical College, Vellore – 632004
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CONSENT FORM FOR PARTICIPATION

Study Title: **Utility of loop- mediated isothermal amplification (LAMP) assay and ELISA in confirmation of leptospirosis**

Study Number: Hospital No:

Subject's Name:

Date of Birth / Age:

(i) I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions.

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

(iii) I understand that the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published.

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

(v) I agree to take part in the above study.

(vi) I also permit the use of the blood sample collected for assessing the usefulness of other tests which may be available in the near future for the detection of this or other related diseases.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative/Guardian: _____ Date:

Signatory's Name: _____

Signature of the Investigator: _____ Date:

Study Investigator's Name: _____

Signature of the Witness: _____ Date:

Name of the Witness: _____

Annexure II

LEPTOSPIROSIS STUDY PROFORMA

Patient Information

Name			Age	Sex	M / F
Address			Contact No		
Hospital No			Unit: med1/ med2/ med3/ med4		
Date Sample Collected			Date Convalescent Sera collected		
Occupation	Veterinary doctor/ sugar cane worker/ rice paddy worker/ abbatoir worker/ dairy farmer/ pet shop/ sanitary worker				
Has pets	Yes/ No		Dogs/ cats/ others		
Exposure to flood	Yes/ No		Family member with leptospirosis		
Had contact with			Animal caracass, excreta, urine		
Exposure to drainage water		Yes/ No	OP	Ward	

Symptoms

Duration of fever	days	Headache	Yes/No	Breathlessness	Yes/No
Extreme weakness	Yes/No	Cough	Yes/No	Rash	Yes/No
Myalgia/Arthralgia	Yes/No	Chills	Yes/No	Calf muscle tenderness	Y/N
Abdominal pain	Yes/No	Jaundice	Yes/No	Decreased urine output	Y/N
Nausea/vomiting	Yes/No	Loose stool	Yes/No	Overt bleeding	Yes/No
Specify	GI/ Haematuria/ Haemoptysis/ Skin/ Gum/ CNS/ Epistaxis/ vaginal				

Co-morbidities

DM	Yes/No	CKD	Yes/No	CVA	Yes/No	Malignancy	Yes/No	none
HTN	Yes/No	CLD	Yes/No	IHD	Yes/No	HIV	Yes/No	

Physical Signs

Pulse		Temperature	
Blood Pressure		Respiratory Rate	
Conjunctival Suffusion	Yes/No	Crepitations	Yes/No
Bilateral Pitting Pedal Oedema	Yes/No	Neck Stiffness	Yes/No
Icterus/ Jaundice	Yes/No	Signs of Dehydration	Yes/No
Abdominal Tenderness	Yes/No	Lymphadenopathy	Yes/No
Hepatomegaly	Yes/No	Splenomegaly	Yes/No
Rash	Yes/No	UL/ LL/ Face/ Neck/ Palms/ Soles	
Macular/Papular/Maculopapular/Vesicular/Petechial/Ecchymotic/Erythematous/Pustular			

Diagnostics on Admission

Haemoglobin		Na/K	/	Bilirubin(D/T)	/
TC		Bicarbonate		SGOT/SGPT	/
Neutrophil	%	Urea/creatinine	/	ALP	
Lymphocyte	%	Amylase/lipase	/	PT/APTT	/
Platelets		ABG pCO_2/O_2	/	SpO_2	
Urine RBC/WBC			pH		
Urine culture			CXR		

Malaria	Pos/neg/nd	Brucella IgM/IgG	Pos/neg/nd	Blood culture	
Widal	Pos/neg/nd	Scrub typhus IgM	Pos/neg/nd	Dengue IgM/IgG	Pos/neg/nd
Spotted fever IgM/IgG		Pos/neg/nd	BBV	HIV/HBsAg/HCV	
ANA		Pos/neg/nd	Pattern	Homo/speckled/nucleolar	

Leptospiral Test

Leptospiral IgM ELISA	Acute	convalescent
Leptospiral PCR		
Leptospiral LAMP		

Course

Antimicrobial		Dose	Duration	
Had Any of the Following		IV Fluid/ Transfusion/ Ventilatory Support/ Pacemaker/ Inotropes/ Dialysis		
Complications		Renal Failure/ ARDS/ Massive Bleeding/ Meningitis/ Myocarditis/ Fulminant Hepatic Failure		
Outcome		Alive/ Dead	Total Stay	
Date Admitted			Date Discharged/died	

Pos- positive

Neg- negative

nd- not done

Study/	Age	Sex	occupation	Address	Fever	abd pain	urine	hmss at	vomiti	myalgia	headach	rash	CNS	chest pai	cough	app	resp dist	bleeding	Platelet	Onset	Bilirubin	Scrub	ARDS	ckosy	ceftra	asd	Improved	ELISA	ELISA	PCR	UAMP	hepta	conv	conv	conv
1	2	0	3	1	2	1	1	0	1	0	0	0	0	0	0	0	0	0	1	2	3	0	0	1	1	0	4	38	1	0	1	0	2	2
2	4	1	2	1	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	2	2	1	0	1	0	1	3	23	1	0	0	2	2
3	2	0	1	1	2	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	2	0	0	1	1	0	0	5	25	1	1	0	2	2
4	1	0	1	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	21	0	1	0	2	2
5	4	0	1	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	1	4	21	1	0	1	0	0
6	4	0	3	1	2	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	3	2	0	1	1	0	1	4	21	1	0	1	1	0
7	3	0	3	1	10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	2	0	1	1	0	0	3	22	1	0	0	2	2
8	1	1	2	1	14	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	4	33	1	0	0	2	2
9	1	1	2	1	5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	3	21	1	0	0	2	2
10	2	0	1	1	10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
11	1	0	1	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
12	5	0	5	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	20	1	0	0	2	2
13	7	1	7	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	25	1	0	0	2	2
14	2	1	2	1	5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	3	20	1	0	0	2	2
15	0	1	2	1	10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	3	21	1	0	0	2	2
16	1	1	2	1	15	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	3	21	1	0	0	2	2
17	1	1	2	1	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	3	21	1	0	0	2	2
18	1	1	2	1	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	1	0	1	1	0	0	3	21	1	0	0	2	2
19	2	0	1	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	1	1	1	0	0	3	21	1	0	0	2	2
20	2	0	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	1	1	1	0	0	3	21	1	0	0	2	2
21	3	0	3	1	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
22	3	0	3	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
23	2	0	3	1	7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
24	3	0	4	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
25	2	0	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
26	4	1	2	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
27	4	0	6	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
28	1	0	5	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
29	4	1	7	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
30	3	0	1	1	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
31	3	0	3	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
32	2	0	3	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
33	2	0	1	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
34	1	1	2	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
35	4	1	3	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
36	3	0	6	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
37	4	0	1	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
38	3	0	3	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
39	3	1	2	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
40	1	1	7	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
41	3	0	1	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
42	4	0	1	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
43	3	0	3	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
44	4	0	7	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
45	2	0	1	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
46	3	1	2	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
47	4	1	2	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
48	3	0	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
49	1	1	5	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	25	1	0	0	2	2
50	2	0	1	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
51	2	0	1	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
52	2	0	3	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
53	4	1	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
54	1	1	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
55	3	0	5	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
56	2	0	4	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
57	2	0	3	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
58	3	0	3	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
59	4	1	2	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
60	2	0	6	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
61	1	0	7	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
62	4	0	6	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
63	1	0	1	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2
64	3	0	1	1	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	1	0	0	3	21	1	0	0	2	2